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David L. Parker

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Lopez-Berestein et al.

Serial No.: 09/982,113

Filed: October 17, 2001

For: A METHOD TO INCORPORATE N-(4-HYDROXYPHENYL) RETINAMIDE IN LIPOSOMES

Group Art Unit: 1615

Examiner: Kishore, Gollamudi S.

Atty. Dkt. No.: UTSC:660US

AMENDED APPEAL BRIEF

MS Appeal Briefs
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

TABLE OF CONTENTS

Sir:

Appellants hereby submit an original and three copies of this Amended Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated February 20, 2004. Appellants filed their original Appeal Brief on July 2, 2004, along with the required fee. Accordingly, it is believe that no fee is due in connection with the filing of this Amended Appeal Brief. However, should any fees be due for any reason related to this filing, the Commissioner is authorized to deduct said fee from Fulbright & Jaworski L.L.P. Account No.: 50-1212/UTSC:660US.

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I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Board of Regents, The University of Texas System, Austin, Texas.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

In response to final, Appellants attempted to amend base claim 54 to focus the present appeal on one of the two alternative embodiments presented by that claim (the "water-containing" embodiment). However, the Examiner refused to enter that narrowing amendment. Therefore, Appellants are proceeding with the present appeal only with respect to dependent claims that present the water-containing embodiment, claims 138-141. It is Appellants' intention, if successful in the present appeal, to introduce amendments into base claim 54 that are consistent with the water-containing embodiment reflected by appealed claims 138-141.

Appellants are concurrently filing a petition with the Group Director requesting reconsideration of the decision not to enter the amendment after final.

A copy of the claims on appeal, claims 138-141, as well as the canceled and withdrawn claims, is attached as Appendix A.

IV. STATUS OF AMENDMENTS

Appellants sought an amendment after final to focus the present appeal on the water-containing embodiments. The Examiner refused to enter this amendment, stating that "the limitations of soybean oil and water are now deleted from claim 54, yet dependent claim 141 recites this limitation." This statement is incorrect in that the after-final amendment did not seek to remove the word "water" from claim 54, it only sought the removal of the words "one or more of soybean oil and," which amendment would have effectively introduced the limitations of claim 138 into claim 54. This was actually stated in the response to final, wherein Appellants stated that:

> Independent claim 54 has been amended and is now directed specifically to the use of 4HPR lipid compositions wherein the lipid composition comprises DMPC and water. Claims 133 and 138 have been cancelled and the dependencies of claims 134 and 139 revised accordingly.
>
> Applicants are amending independent claim 54 in order to prosecute the claimed subspecies of allowance/appeal. Applicants reserve the right to proceed with claims directed to the additional subject matter in future continuing applications. If the pending claims are passed to allowance, Applicants would propose to cancel withdrawn claims 61-119 and 130, or authorize an examiner's amendment, in order to place the case into condition for allowance.

The Examiner was subsequently contacted by phone and Appellant's representative attempted to explain the appropriateness of the amendment, but the Examiner again refused to enter the amendment.

Subsequently, special examiner Deborah Reynolds was contacted and she advised that she conferred with Examiner Kishore and SPE Page and that she concluded they were correct in their determination that the amendments sought to independent claim 54 actually broadened the claim. We continue to disagree and, as noted above, have petitioned the Group Director.

V. SUMMARY OF THE INVENTION

The invention that is the subject of the present appeal is directed to the treatment of cancer using a compound known as N-(4-hydroxyphenyl) retinamide or a derivative thereof (hereinafter collectively "4-HPR") that is formulated in a composition that includes the phospholipid DMPC and water, as well as possibly other elements such as soybean oil. The subject matter of the present appeal is reflected generally by dependent claim 138, which can be depicted in independent form as follows:

> 138. A method of treating a subject having cancer, comprising administering to said individual a therapeutically effective amount of a composition comprising N-(4-hydroxyphenyl) retinamide, or a derivative thereof, encapsulated in a lipid material, wherein said lipid material comprises dimyristoyl phosphatidylcholine (DMPC) and water.

Dependent claims 139 and 140 make reference to specific percentages and ranges of water, whereas claim 141 refers to the inclusion of DMPC, water and soybean oil.

VI. ISSUES ON APPEAL

The issues addressed in this appeal include:

a) Whether the subject matter of claims 138-141 is obvious over the combination of Mehta, US 5,811,119 (Exhibit 1) in view of Ulukaya (Exhibit 2);

b) Whether the subject matter of claims 138-141 is obvious over the combination of Mehta in view of Minton, US 5,008,291 (Exhibit 3) or Zeligs, US 6,093,706 (Exhibit 4), or vice versa.

VII. GROUPING OF THE CLAIMS

The claims are to be considered separately, and separate arguments are presented hereinbelow.

VIII. ARGUMENT

A. The rejection of claims 138-141 over Mehta in view of Ulukaya

1. Summary of Rejection

The Action rejects claims 138-141 over the combination of Mehta in view of Ulukaya, taking the position that Mehta teaches the use of liposomal retinoids comprising soybean oil and DMPC in the treatment of cancer. Ulukaya is cited as teaching that 4-HPR is a retinoid known to be useful in treating cancer.

The Final Action included no reference to any teaching in either Mehta or Ulukaya that concerns the inclusion of water in the liposomes of Mehta. When this pointed out in Appellants' response to final, the Advisory Action stated merely that "applicant's arguments that Mehta's liposomes do not have water are not found to be persuasive since liposomal bilayer formation occurs only after hydration with water." The Examiner provided no teaching to support this conclusion which, for the reasons stated below, is incorrect.

2. Appellants' Remarks

a) Substantial evidence required to uphold the examiner's position

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

b) The standard for obviousness

In order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. *Manual of Patent Examining Procedure* § 2142. Moreover, the teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on Applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991). When "the motivation to combine the teachings of the references is not immediately apparent, it is the duty of the examiner to explain why the combination of the teachings is proper." MPEP § 2142.

c) No Proper Prima Facie Rejection Made

It is Appellant's position that the Examiner has failed to make out a *prima facie* obviousness rejection over the references, alone or in combination, which have not been shown to teach or suggest each of the elements of the claims. The principal reference, Mehta, fails to teach or suggest the use of DMPC and water to form its liposomes. Mehta's Example 1 describes the preparation of liposomal-all trans-retinoic acid ("L-RA"). In the paragraph beginning at col 7, line 54 of Mehta, it is described that the retinoic acid ("RA") is comprised in t-butanol, and that the butanol-solubilized RA is then added to the "dried lipid film" to form the liposomes. It is specifically stated that only butanol, ***not*** butanol + water, was used to form the

liposomal retinoid. Thus, there is no teaching here that water or an aqueous solution is added during the liposomal formulation step.

The Examiner apparently relies upon the statement in Mehta which concerns "reconstitution" of already-formed "liposomal retinoic acid" in an aqueous solution (see, *e.g.*, col. 7, ln 66, to col. 8, ln 3). The Examiner has not shown that resuspending already-formed liposomes in an aqueous solution results in the introduction of water into the lipid layer – in fact, it does not! Resuspending already-formed liposomal/drug in an aqueous results in water going into the interior of the liposome, not into the lipid bilayer. It is noted that the claims require that the "lipid material" used to form the liposomes comprise water. In contrast to the liposomes of Mehta, the present liposomes actually incorporate water in the lipid bilayer by virtue of it's presence in the starting butanol. As explained below and exemplified in the present specification, inclusion of water in the lipid material at the time of formation of the liposome has significant advantages over the Mehta approach of not including water in the lipid material

In contrast, Applicants' specification discloses, for example in Example 1 and Table 2 (page 83; attached as Exhibit 5 hereto), that liposomal 4-HPR can be formed using DMPC and water, or DMPC + water + soybean oil ("SO"), and, as explained in more detail below, that such water containing formulations have surprisingly higher encapsulation efficiencies, at least in the context of 4-HPR, than do 4-HPR lipid formulations prepared without the inclusion of water.

d) The secondary reference does not cure the defect

None of the secondary references contains a teaching that remedies the deficiencies in Mehta with respect to the inclusion of water in the DMPC phospholipid composition used to form liposomes. Ulukaya contains no disclosure that Applicants can find that suggests a liposomal formulation, and the Examiner appears to concede this and simply relies on supposed advantages of 4-HPR *per se*. However, it is unclear how this fact alone provides a suggestion to

provide the 4-HPR or water in the lipid formulation of Mehta. Indeed, there is evidence in Ulukaya that teaches away from the combination: Ulukaya teaches that 4-HPR has properties that distinguish it from naturally occurring retinoids, including the fact that it apparently exerts its clinical effects by a different pathway from classical retinoids. This fact suggests that 4-HPR has *different* physicochemical and/or biological properties from classical retinoids, which immediately brings into question whether one of ordinary skill would have an expectation that this very different retinoid could be practiced in the context of the teachings of Mehta. We think not. Furthermore, with the known advantages described in Ulukaya, we question whether one of skill would be motivated to try to modify it in any way. Again, we think not.

e) *Claims 139 and 140*

The subject matter of claims 139 and 140 are separately patentable. Claim 139 is directed to a composition wherein the lipid material comprises from 1 to 10% water, and claim 140 is directed to a composition wherein the lipid material comprises about 10% water. The Examiner has not even attempted to make a *prima facie* rejection of the subject matter of these claims. As noted below in the secondary considerations section, such formulations have surprisingly high 4-HPR encapsulation efficiency, particularly when compared to liposomes prepared without water as taught by Mehta.

f) *Evidence of Secondary Considerations*

As noted above, there exists here strong evidence to support a conclusion of non-obviousness. Applicants' specification discloses, in Example 1 and Table 2 (page 83; attached as Exhibit 5 hereto), that liposomal 4-HPR can be formed using DMPC and water, or DMPC + water + soybean oil ("SO"). Here, the water in included in the t-butanol that is employed for solubilizing the lipids and 4-HPR to form liposomes. Importantly, the encapsulation efficiency achieved by the present inventors for 4-HPR using water or water + SO was consistently very

high: 81.5% with DMPC + water and ranging from 77.5% up to 96.4% with DMPC + water + SO. This was in direct contrast to the much poorer 60% encapsulation efficiency achieved without the inclusion of water, which is reflective of the approach taught by Mehta.

g) Claims 138-141

The data set forth in Table 2 demonstrates that formulations made with DMPC + water have a surprisingly higher 4-HPR encapsulation efficiency than formulations made with DMPC and no water (81.5% vs. 60.0%). Similarly, it is shown that increasingly higher amounts of water provide an increasingly higher incorporation efficiency (77.5%, 81.5% and 87.5% for lower amounts of water and 88.3%, 92.8% and 96.4% for higher amounts of water). This is particularly relevant to the patentability of claims 138-140.

Furthermore, the data demonstrates that the inclusion of all three of DMPC + soybean oil + water is even more preferred than either DMPC + water alone, or DMPC alone (average 88.5% vs. 81.5% vs. 60.0%). This is particularly relevant to the patentability of claim 141.

B. The rejection of claims 138-141 over Mehta in view of Minton or Zeligs

1. Summary of Rejection

The Final Action next rejects claims, including claims 138-141, as obvious over Mehta in view of Zeligs. The rejection is essentially as set forth above for the foregoing rejection, with the exception that the secondary references of Minton and Zeligs are relied upon. Minton is said to teach 4-HPR in the treatment of cancer and is also said to teach the use of sustained or continuous release formulations (col. 13, lns 17-18). Zeligs is also said to teach 4-HPR in the treatment of cancer and is said to teach administration in the form of liposomes (col. 6, ln 60).

2. Appellants' Remarks

First of all, Appellants incorporate by reference all of the foregoing comments with respect to the rejection over Mehta in view of Ulukaya. Further with respect to the two

additional references of Minton and Zeligs, Appellants note that neither of these references appear to teach or suggest the use of water in the lipids used to form liposomal 4-HPR and thus do not appear to address the shortcomings noted above.

The Minton reference is said to teach sustained or continuous release formulations, but it is hard to see how this disclosure is relevant to DMPC/SO/water formulations of 4-HPR and the Examiner has not provided any explanation in this regard. On the contrary, Minton simply teaches that one can prepare sustained release formulations of the 4-HPR and calcium glucarate. However, it is hard to imagine how the preparation of a sustained release formulation of these two drugs would lead one of skill in the art to Mehta – as noted above, Mehta is concerned with formulations having reduced toxicity and appears to teach that one can administer liposomal retinoids for longer periods of time without toxicity (see, *e.g.*, col. 4). Perhaps Appellants have missed the teachings that the Examiner is relying upon. So, if the Examiner is aware of some teaching in Mehta that its liposomal retinoid formulations are for the purpose of providing a sustained release formulation, the Examiner is requested to identify the teaching relied upon on the record. (Applicants have on-line searched the Mehta patent for the word "sustained" without success, and have only found the word "continuous" in relation to continuous therapy with retinoids as opposed to teaching that the lipid formulations provide this benefit.)

Zeligs is the one reference that does refer generically to liposomal formulations of DHEA and retinoids such as 4-HPR, but again, there is no basis for combining this teaching with Mehta *per se* to arrive at the presently claimed invention. In particular, it is noted that Zeligs is primarily concerned with topical therapy and topical compositions for the treatment of skin disorders and for protection against UV light. There is some disclosure that concerns parenteral administration – indeed, liposomal formulations are only mentioned in the context of systemic administration (col. 6, ln 60). While no specific indications for liposomal formulations *per se* are

given by Zeligs (see col. 6, ln 60), there is disclosure that systemic administration is to "prevent" the "recurrence" of squamous cell carcinoma. Furthermore, there is no disclosure that would, in the Applicants' opinion, suggest to one of skill in the art to select and use a DMPC/SO/water formulations – certainly a very general disclosure such as Zeligs (which only appears to mention the word "liposome" once) cannot render each and every cancer therapeutic methods suing lipid formulations obvious.

IX. CONCLUSION

Appellants have provided arguments that overcome the pending rejections. Appellants respectfully submit that the Final Official Action's conclusions that the claims should be rejected are unwarranted. It is therefore requested that the Board overturn the Final Action's rejections.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Respectfully submitted,

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APPENDIX A

1. – 53. (Canceled)

54. (Canceled) A method of treating a subject having cancer, comprising administering to said individual a therapeutically effective amount of a composition comprising N-(4-hydroxyphenyl) retamide, or a derivative thereof, encapsulated in a lipid material, wherein said lipid material comprises dimyristoyl phosphatidylcholine (DMPC) and one or more of soybean oil (SO) and water.

55. (Canceled) The method of claim 54, wherein said dimyristoyl phosphatidylcholine and soybean oil comprise a ratio of greater than 80:20.

56. (Canceled) The method of claim 54, wherein said composition is comprised in a pharmaceutically acceptable aqueous medium.

57. (Canceled) The method of claim 54, wherein said method further comprises administering at least one additional therapeutic agent to said individual.

58. (Canceled) The method of claim 57, wherein said agent is comprised in said composition.

59. (Canceled) The method of claim 57, wherein said additional therapeutic agent comprises at least one anticancer agent.

60. (Canceled) The method of claim 59, wherein the anticancer agent is chemotherapy agent, a radiotherapy agent, an immune therapy agent, a genetic therapy agent, a hormonal therapy agent, a biological agent, an additional retinoid or a retinoid derivative.

61. (Withdrawn) A method for increasing growth inhibitory effects of fenretinide on a cell comprising providing to a cell, in combination with fenretinide, one or more agents that increases the level of nitric oxide (NO) in said cell.

62. (Withdrawn) The method of claim 61, wherein said cell is a tumor cell.

63. (Withdrawn) The method of claim 62, wherein said tumor cell is a breast cancer cell.

64. (Withdrawn) The method of claim 63, wherein the breast cancer cell is an estrogen receptor (ER)-positive cell.

65. (Withdrawn) The method of claim 63, wherein the breast cancer cell is an estrogen receptor (ER)-negative cell.

66. (Withdrawn) The method of claim 61, wherein fenretinide is provided before the one or more agents.

67. (Withdrawn) The method of claim 61, wherein fenretinide is provided at the same time as the one or more agents.

68. (Withdrawn) The method of claim 61, wherein fenretinide is provided after the one or more agents.

69. (Withdrawn) The method of claim 61, wherein fenretinide is provided more than once.

70. (Withdrawn) The method of claim 69, wherein fenretinide is provided daily for three months with monthly three-day interruptions.

71. (Withdrawn) The method of claim 61, wherein said agent is provided more than once.

72. (Withdrawn) The method of claim 61, wherein said agent is a nucleic acid.

73. (Withdrawn) The method of claim 72, wherein said nucleic acid is an expression construct encoding iNOS, interferon-γ or herceptin.

74. (Withdrawn) The method of claim 61, wherein said agent is a protein.

75. (Withdrawn) The method of claim 74, wherein said protein is iNOS, interferon-γ or herceptin.

76. (Withdrawn) The method of claim 61, wherein said agent is a chemopharmaceutical.

77. (Withdrawn) The method of claim 76, wherein said agent is cyclosporin A.

78. (Withdrawn) The method of claim 62, wherein said cell tumor cell is a patient.

79. (Withdrawn) The method of claim 78, wherein said cell tumor cell is part of a tumor mass in said patient.

80. (Withdrawn) The method claim 78, wherein providing comprises direct administration to said tumor cell.

81. (Withdrawn) The method of claim 61, further comprising providing to said cell an additional anti-cancer therapy.

82. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is radiation.

83. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is a distinct chemotherapy.

84. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is a distinct gene therapy.

85. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is immunotherapy.

86. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is hormonal therapy.

87. (Withdrawn) The method of claim 61, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration of 0.1 μm.

88. (Withdrawn) The method of claim 61, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration of 0.5 μm.

89. (Withdrawn) The method of claim 61, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration of 1.0 μm.

90. (Withdrawn) The method of claim 61, wherein said cell is killed.

91. (Withdrawn) A method for treating cancer in a subject comprising providing to said subject, in combination, fenretinide and one or more agents that increases the level of nitric oxide (NO) in cancer cells in said subject.

92. (Withdrawn) The method of claim 91, wherein said cancer is a breast cancer.

93. (Withdrawn) The method of claim 92, wherein cells of said breast cancer are estrogen receptor (ER)-positive.

94. (Withdrawn) The method of claim 92, wherein cells of said breast cancer are estrogen receptor (ER)-negative.

95. (Withdrawn) The method of claim 91, wherein fenretinide is provided before the one or more agents.

96. (Withdrawn) The method of claim 91, wherein fenretinide is provided at the same time as the one or more agents.

97. (Withdrawn) The method of claim 91, wherein fenretinide is provided after the one or more agents.

98. (Withdrawn) The method of claim 91, wherein fenretinide is provided more than once.

99. (Withdrawn) The method of claim 98, wherein fenretinide is provided daily for three months with monthly three-day interruptions.

100. (Withdrawn) The method of claim 91, wherein said agent is provided more than once.

101. (Withdrawn) The method of claim 91, wherein said agent is a nucleic acid.

102. (Withdrawn) The method of claim 101, wherein said nucleic acid is an expression construct encoding iNOS, interferon-γ or herceptin.

103. (Withdrawn) The method of claim 91, wherein said agent is a protein.

104. (Withdrawn) The method of claim 103, wherein said protein is iNOS, interferon-γ or herceptin.

105. (Withdrawn) The method of claim 91, wherein said agent is a chemopharmaceutical.

106. (Withdrawn) The method of claim 105, wherein said agent is cyclosporin A.

107. (Withdrawn) The method claim 91, wherein providing comprises direct administration to said tumor cell.

108. (Withdrawn) The method of claim 91, further comprising providing to said cell an additional anti-cancer therapy.

109. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is radiation.

110. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is a distinct chemotherapy.

111. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is a distinct gene therapy.

112. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is immunotherapy.

113. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is hormonal therapy.

114. (Withdrawn) The method of claim 91, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration in cancer cells of 0.1 μm.

115. (Withdrawn) The method of claim 91, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration in cancer cells of 0.5 μm.

116. (Withdrawn) The method of claim 91, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration in cancer cells of 1.0 μm.

117. (Withdrawn) The method of claim 91, wherein fenretinide is provided at 10 mg/day.

118. (Withdrawn) The method of claim 91, wherein fenretinide is provided at 100 mg/day.

119. (Withdrawn) The method of claim 91, wherein fenretinide is provided at 200 mg.day.

120. – 129. (Cancelled)

130. (Withdrawn) A method for inhibiting metastasis in a subject having cancer comprising providing to said subject, in combination, fenretinide and one or more agents that increases the level of nitric oxide (NO) in cancer cells in said subject.

131. (Canceled) The method of claim 54, wherein the composition is administered parenterally to the individual.

132. (Canceled) The method of claim 54, wherein the composition is administered orally to the individual.

133. (Canceled) The method of claim 54, wherein the lipid material comprises DMPC and SO.

134. (Canceled) The method of claim 133, comprising a ratio of 4-HPR, or derivative thereof, to DMPC/SO of from 1:5 to 1:15.

135. (Canceled) The method of claim 134, wherein the 4-HPR, or derivative thereof, to DMPC/SO ratio is about 1:5 (w/w).

136. (Canceled) The method of claim 134, wherein the 4-HPR, or derivative thereof, to DMPC/SO ratio is about 1:10 (w/w).

137. (Canceled) The method of claim 134, wherein the 4-HPR, or derivative thereof, to DMPC/SO ratio is about 1:15 (w/w).

138. (Currently amended) A method of treating a subject having cancer, comprising administering to said individual a therapeutically effective amount of a composition comprising N-(4-hydroxyphenyl) retamide, or a derivative thereof, encapsulated in a lipid material, ~~The method of claim 54,~~ wherein the lipid material comprises dimyristoyl phosphatidylcholine (DMPC) and water.

139. (Previously presented) The method of claim 138, wherein the composition comprises from 1 to 10% water.

140. (Previously presented) The method of claim 139, wherein the composition comprises about 10% water.

141. (Currently amended) A method of treating a subject having cancer, comprising administering to said individual a therapeutically effective amount of a composition comprising N-(4-hydroxyphenyl) retamide, or a derivative thereof, encapsulated in a lipid material, ~~The method of claim 54,~~ wherein the lipid material comprises dimyristoyl phosphatidylcholine (DMPC), soybean oil (SO) and water.

US005811119A

United States Patent [19]

Mehta et al.

[11] Patent Number: 5,811,119

[45] Date of Patent: Sep. 22, 1998

[54] FORMULATION AND USE OF CAROTENOIDS IN TREATMENT OF CANCER

[75] Inventors: **Kapil Mehta; Roman Perez-Soler; Gabriel Lopez-Berestein**, all of Houston; **Robert P. Lenk**, Willis; **Alan C. Hayman, deceased**, late of Houston, all of Tex., by Katherine J. Hayman, legal representative

[73] Assignees: **Board of Regents, the University of Texas**, Austin; **Aronex Pharmaceuticals, Inc.**, The Woodlands, both of Tex.

[21] Appl. No.: **735,310**

[22] Filed: **Oct. 22, 1996**

Related U.S. Application Data

[63] Continuation of Ser. No. 286,928, Aug. 8, 1994, abandoned, which is a continuation-in-part of Ser. No. 213,249, Mar. 14, 1994, abandoned, which is a continuation of Ser. No. 822,055, Jan. 16, 1992, abandoned, which is a continuation-in-part of Ser. No. 588,143, Sep. 25, 1990, abandoned, which is a division of Ser. No. 152,183, Feb. 4, 1988, abandoned, which is a continuation-in-part of Ser. No. 51,890, May 19, 1987, Pat. No. 4,863,739.

[51] **Int. Cl.**[6] **A61K 9/127**

[52] **U.S. Cl.** **424/450**

[58] **Field of Search** 424/450; 514/725

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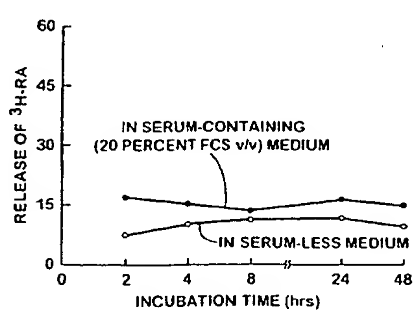
Primary Examiner—Gollamudi S. Kishore
Attorney, Agent, or Firm—Arnold, White & Durkee

[57] **ABSTRACT**

A reduced-toxicity formulation of carotenoids is disclosed which is stable in an aqueous environment. The formulation includes a carotenoid, lipid carrier particles (such as liposomes), and an intercalation promoter agent (such as a triglyceride), which causes the carotenoid to be substantially uniformly distributed with the lipid in the lipid carrier particles. The molar ratio of carotenoid to lipid is greater than about 1:10. Also disclosed is a method of inhibiting the growth of cancer cells, which comprises administering to a living subject a therapeutically effective amount of a composition as described above.

2 Claims, 9 Drawing Sheets

STABILITY OF ^{3} H-RETINOIC ACID IN PRESENCE OR ABSENCE OF SERUM PROTEINS AFTER ENCAPSULATION IN LIPOSOMES.



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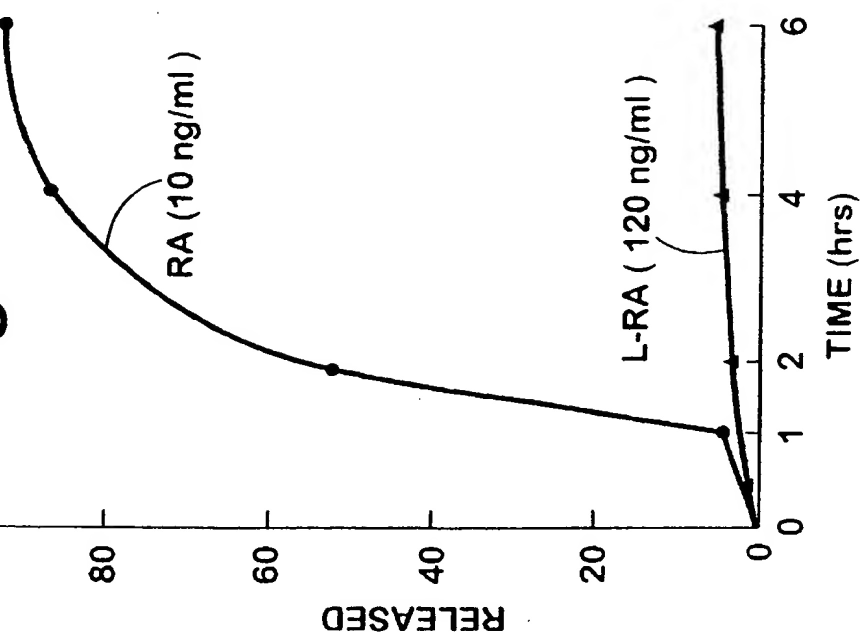
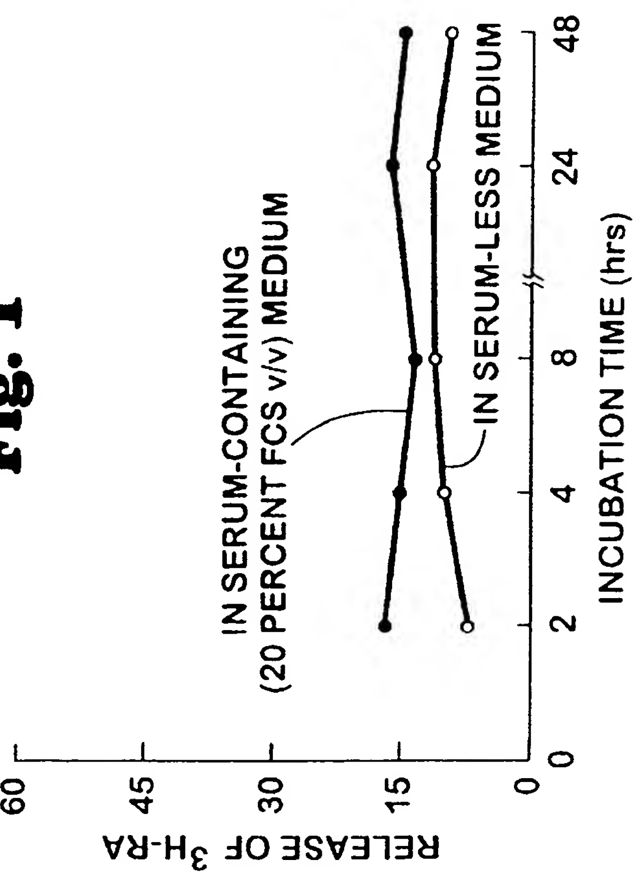


Fig. 2

STABILITY OF ^{3} H-RETINOIC ACID IN PRESENCE OR ABSENCE OF SERUM PROTEINS AFTER ENCAPSULATION IN LIPOSOMES.

Fig. 1



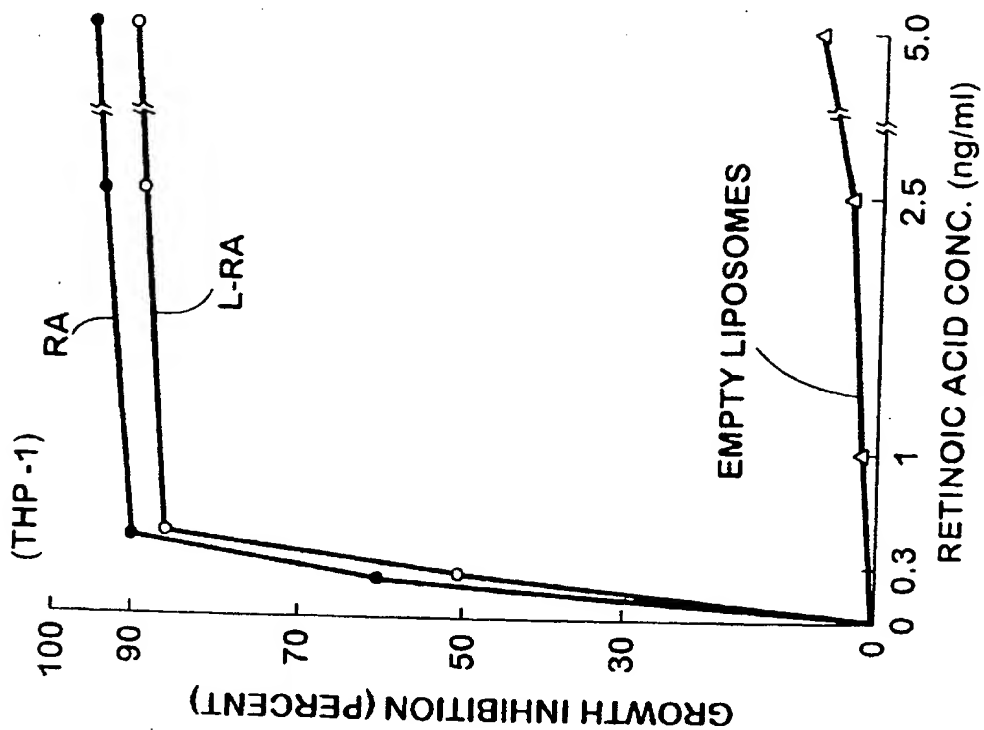

(THP-1)
RA
L-RA
EMPTY LIPOSOMES
GROWTH INHIBITION (PERCENT)
100
90
70
50
30
0
0
0.3
1
2.5
5.0
RETINOIC ACID CONC. (ng/ml)

Fig. 4

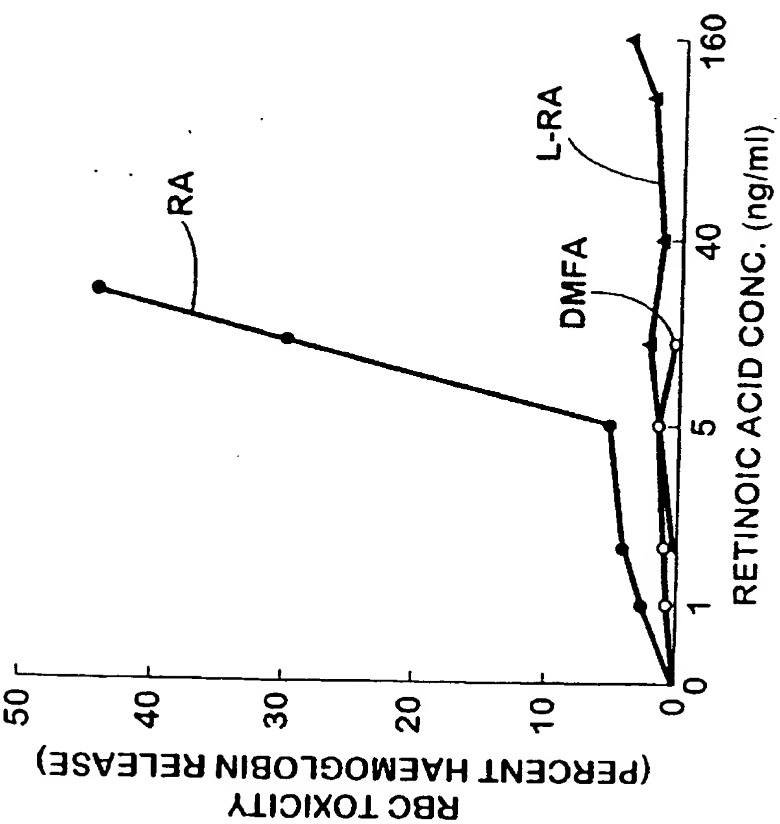

RA
DMFA
L-RA
RBC TOXICITY
(PERCENT HAEMOGLOBIN RELEASE)
50
40
30
20
10
0
0
1
5
40
160
RETINOIC ACID CONC. (ng/ml)

Fig. 3

Fig. 6

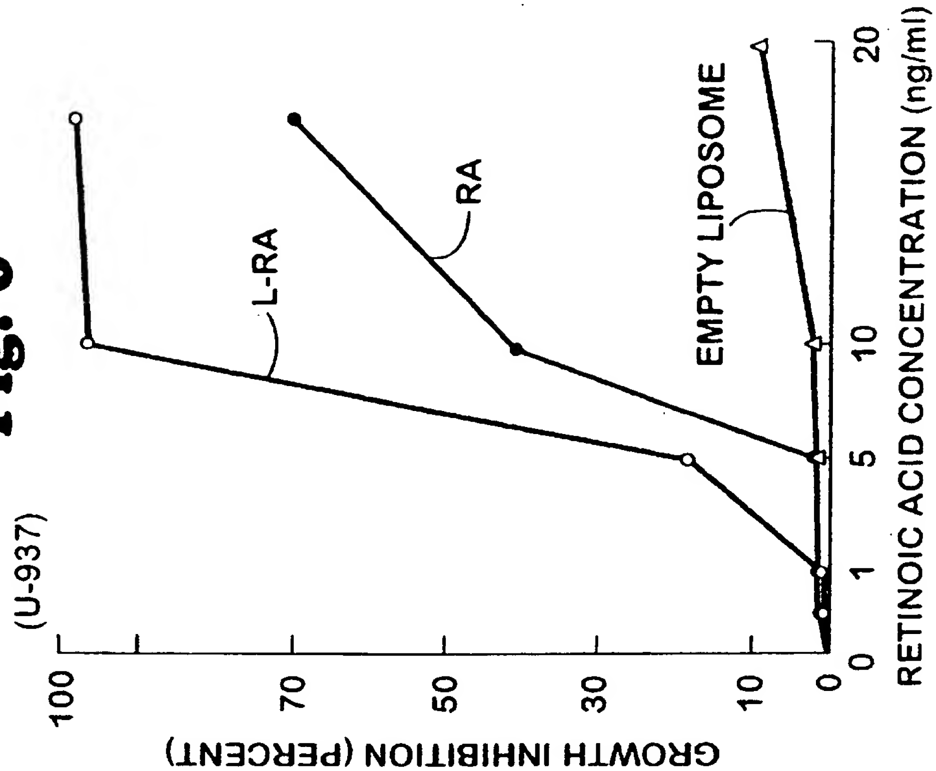

(U-937)
GROWTH INHIBITION (PERCENT)
100
70
50
30
10
0
0
1
5
10
20
RETINOIC ACID CONCENTRATION (ng/ml)
L-RA
RA
EMPTY LIPOSOME

Fig. 5

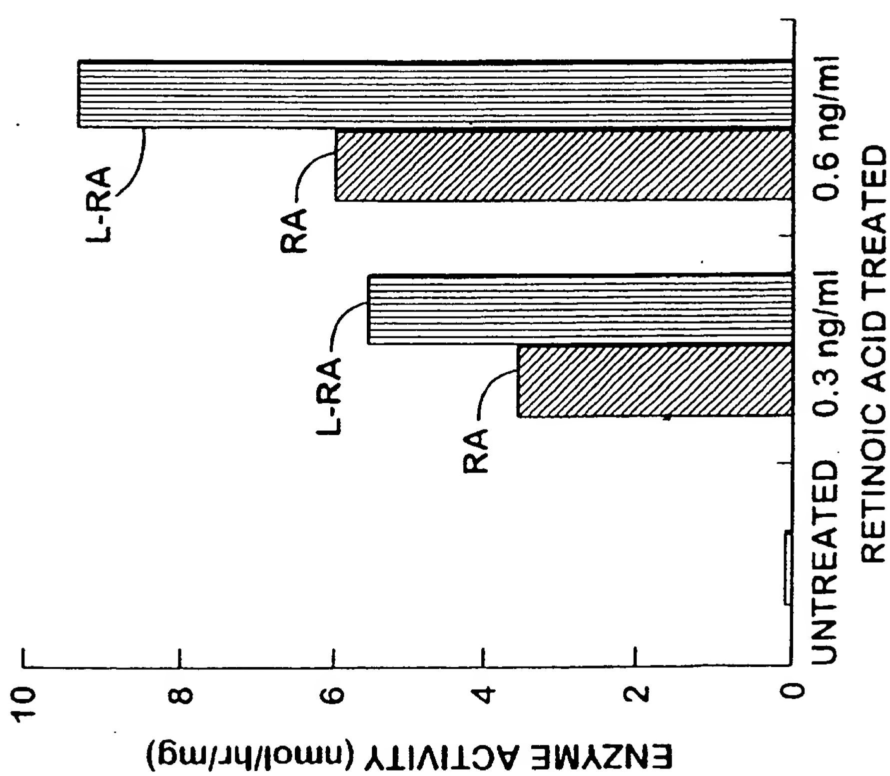

ENZYME ACTIVITY (nmol/hr/mg)
10
8
6
4
2
0
UNTREATED
0.3 ng/ml
0.6 ng/ml
RETINOIC ACID TREATED
RA
L-RA
RA
L-RA

Fig. 8

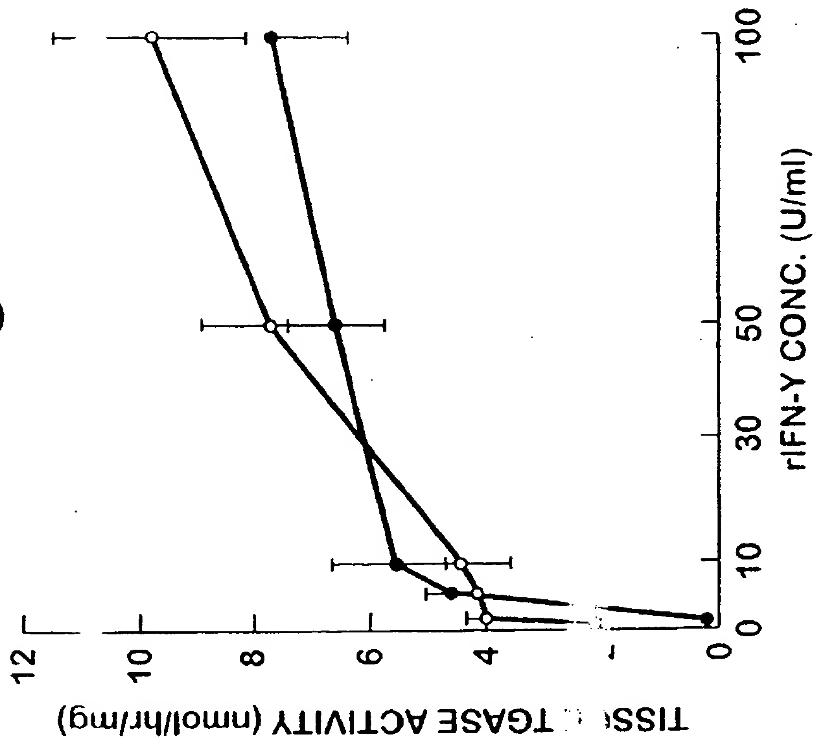
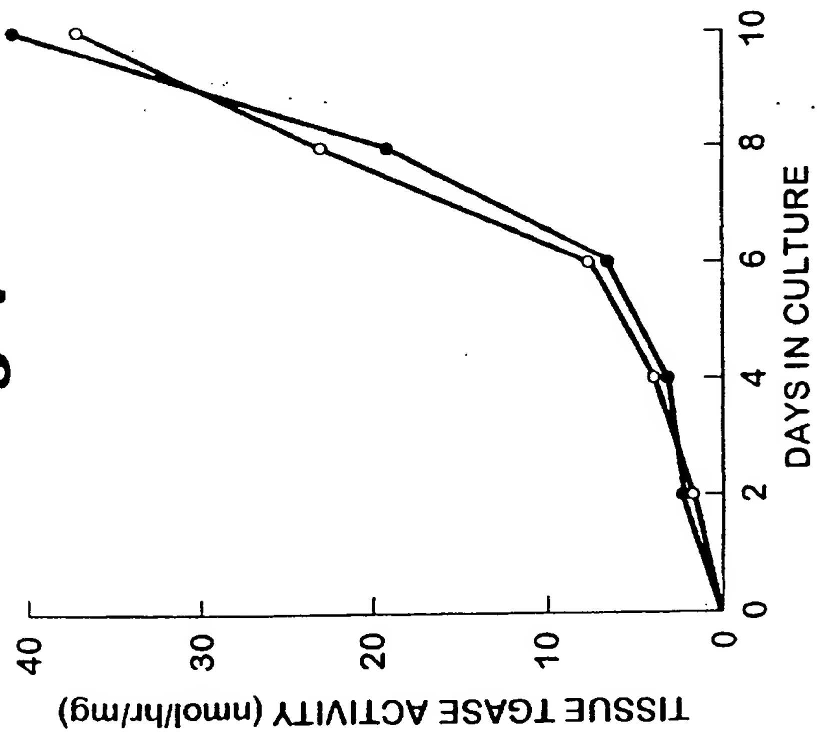

TGASE ACTIVITY (nmol/hr/mg)
0
4
6
8
10
12
rIFN-Y CONC. (U/ml)
0
10
30
50
100

Fig. 7


TISSUE TGASE ACTIVITY (nmol/hr/mg)
0
10
20
30
40
DAYS IN CULTURE
0
2
4
6
8
10

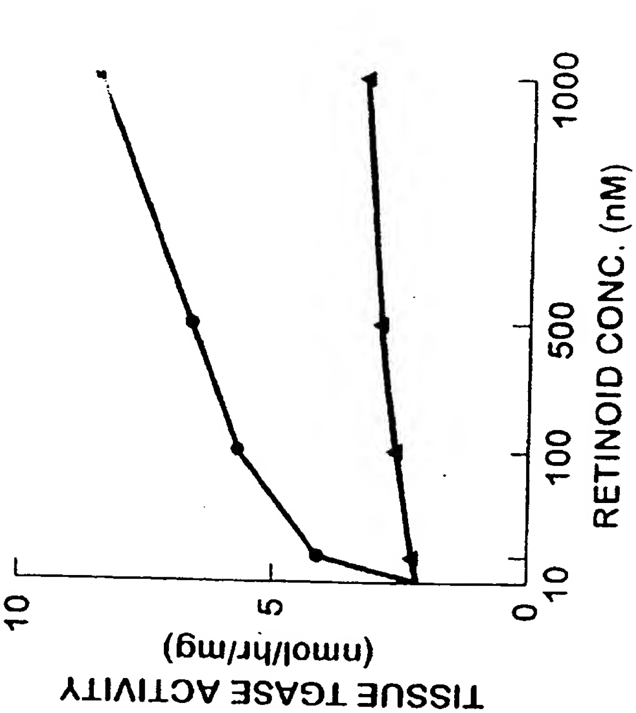

TISSUE TGASE ACTIVITY
(nmol/hr/mg)
10
5
0
10
100
500
1000
RETINOID CONC. (nM)

Fig. 9B

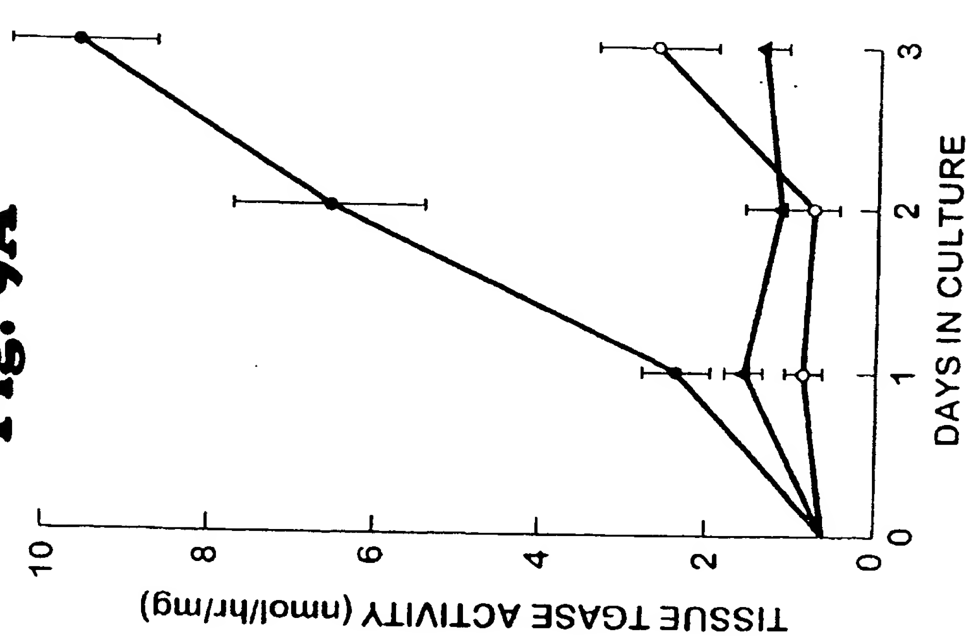

TISSUE TGASE ACTIVITY (nmol/hr/mg)
10
8
6
4
2
0
0
1
2
3
DAYS IN CULTURE

Fig. 9A

Fig. 10A

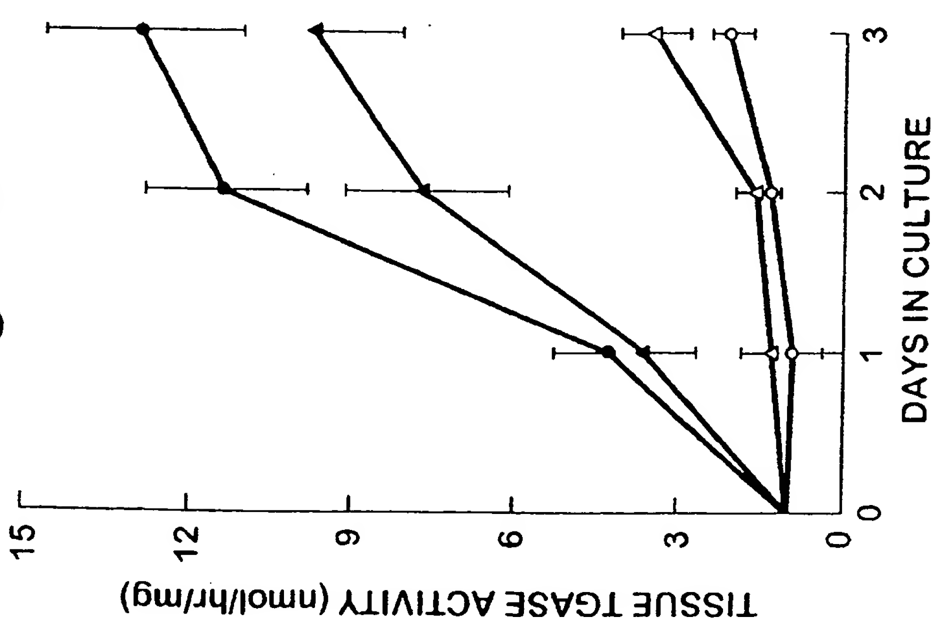


Fig. 11A

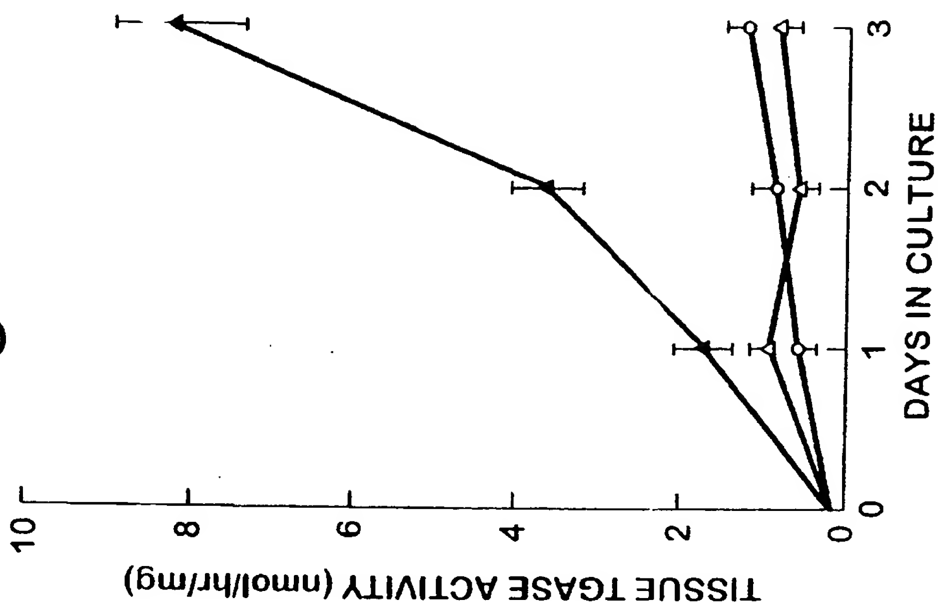


Fig. 10B

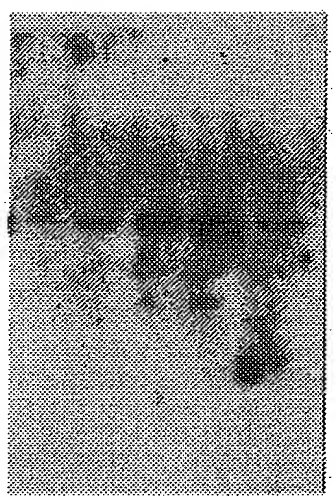


Fig. 11B

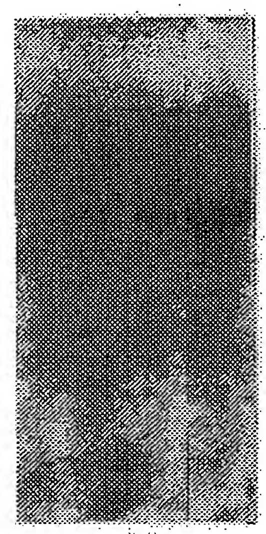


Fig. 12B

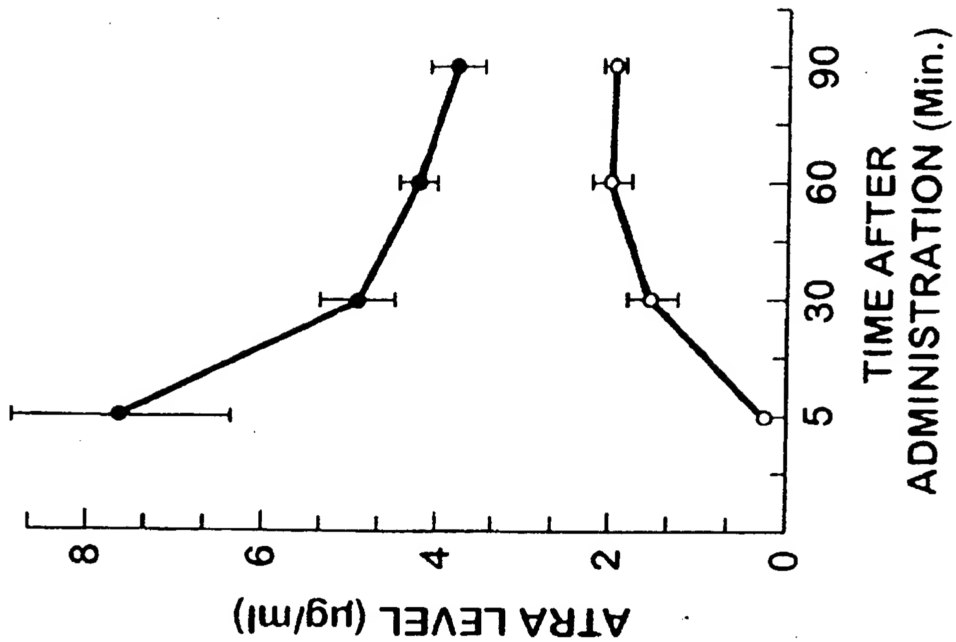
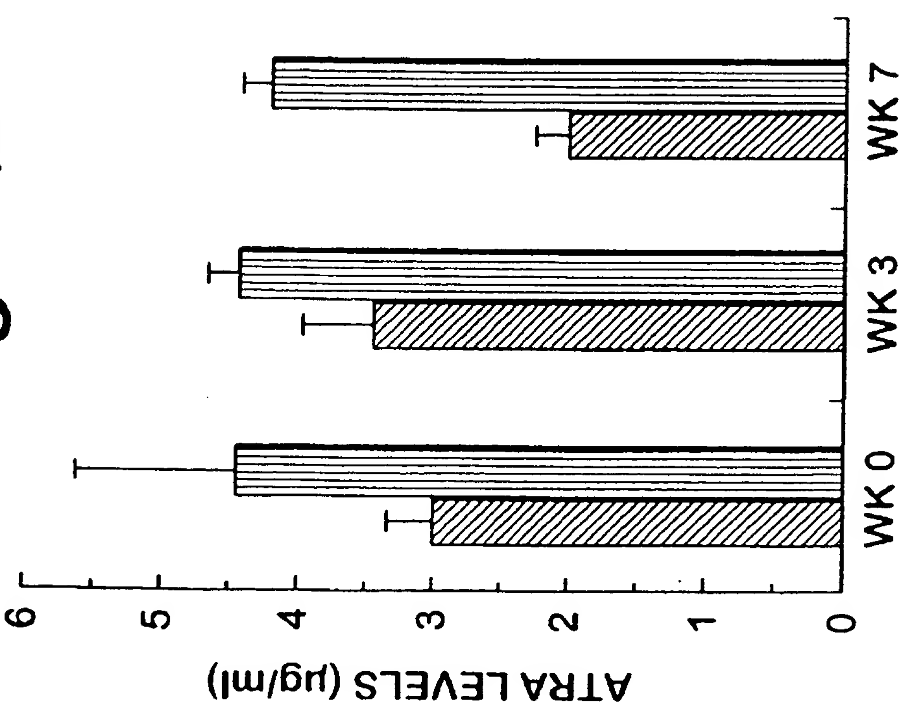


Fig. 12A



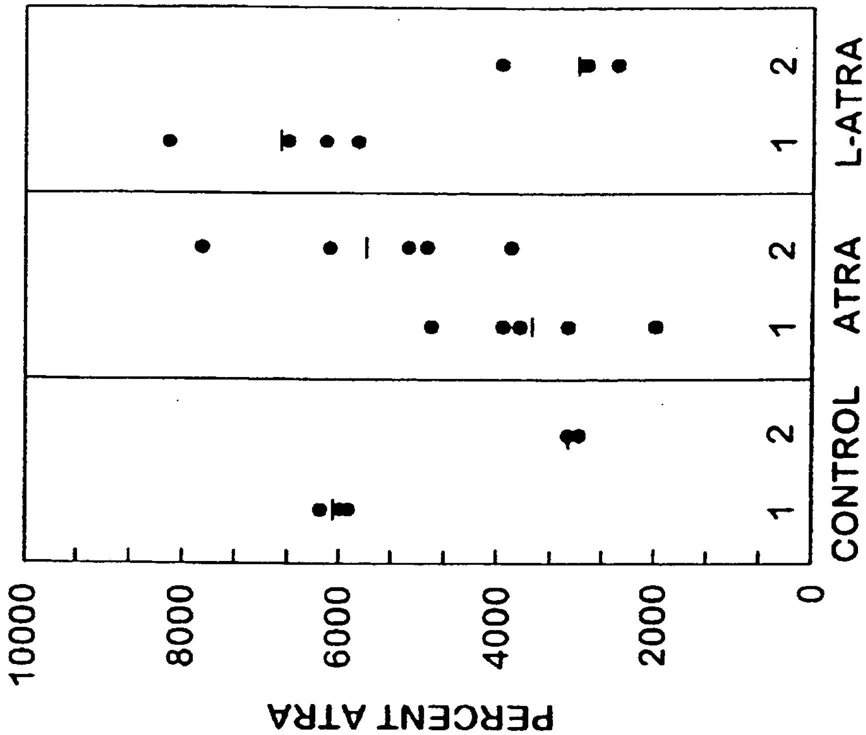


Fig. 13B

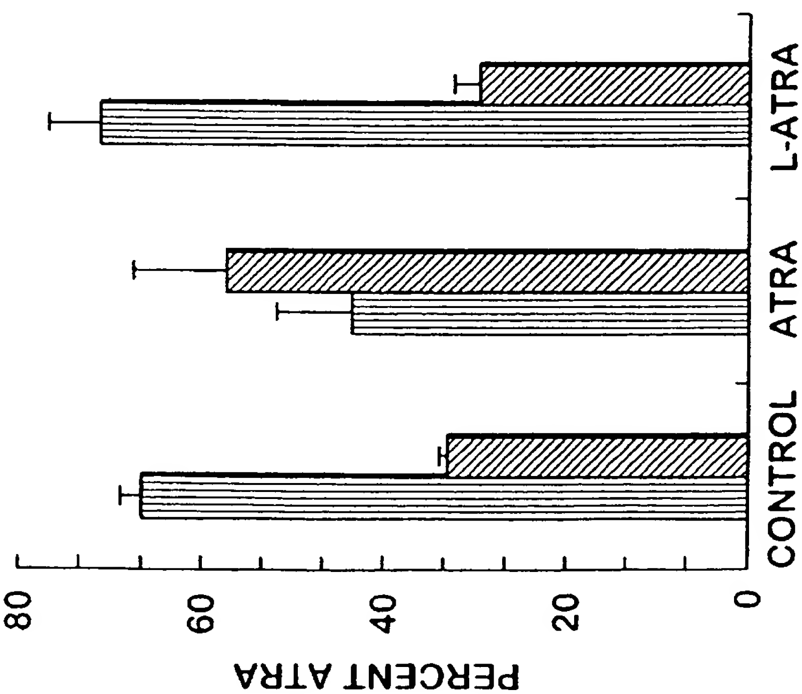


Fig. 13A

FORMULATION AND USE OF CAROTENOIDS IN TREATMENT OF CANCER

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Ser. No. 08/286,928, filed on Aug. 8, 1994, now aban. which is a continuation-in-part of U.S. Ser. No. 08/213,249, filed on Mar. 14, 1994 now abandoned, which is a continuation of U.S. Ser. No. 07/822,055, filed on Jan. 16, 1992, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/588,143, filed on Sep. 25, 1990, now abandoned, which is a divisional of U.S. Ser. No. 07/152,183, filed on Feb. 4, 1988, now abandoned. The 152,183 application is also a continuation in part of U.S. Ser. No. 051,890, filed on May 19, 1987, issued as U.S. Pat. No. 4,863,739, Sep. 5, 1989. The above-identified applications are incorporated here by reference.

BACKGROUND OF THE INVENTION

The present invention relates to therapeutic compositions of carotenoids encapsulated in liposomes or other lipid carrier particles.

It has been known for more than 50 years that retinoids, the family of molecules comprising both the natural and synthetic analogues of retinol (vitamin A), are potent agents for control of both cellular differentiation and cellular proliferation (Wolbach et al., J. Exp. Med., 42:753–777, 1925). Several studies have shown that retinoids can suppress the process of carcinogenesis in vivo in experimental animals (for reviews, see e.g., Bollag, Cancer Chemother. Pharmacol., 3:207–215, 1979, and Sporn et al., In Zedeck et al. (eds.), Inhibition of Tumor induction and development, pp. 71–100. New York: Plenum Publishing Corp., 1981). These results are now the basis of current attempts to use retinoids for cancer prevention in humans. Furthermore, there is extensive evidence which suggests that retinoids can suppress the development of malignant phenotype in vitro (for review, see e.g., Bertram et al., In: M. S. Arnott et al., (eds.), Molecular interactions of nutrition and cancer, pp 315–335. New York, Raven Press, 1982; Lotan et al., The modulation and mediation of cancer by vitamins, pp 211–223. Basel: S. Karger AG, 1983) thus suggesting a potential use of retinoids in cancer prevention. Also, recently it has been shown that retinoids can exert effects on certain fully transformed, invasive, neoplastic cells leading in certain instances to a suppression of proliferation (Lotan, Biochim. Biophys. Acta, 605:33–91, 1980) and in other instances to terminal differentiation of these cells, resulting in a more benign, non-neoplastic phenotype (see e.g., Brietman et al., Proc. Natl. Acad. Sci. U.S.A., 77:2936–2940, 1980).

Retinoids have also been shown to be effective in the treatment of cystic acne (see e.g., Peck, et al., New Engl. J. Med., 300:329–333, 1979). In addition to cystic acne, retinoid therapy has been shown to be effective in gram-negative folliculitis, acne fulminans, acne conglobata, hidradenitis suppurativa, dissecting cellulitis of the scalp, and acne rosacea (see e.g., Plewig et al., J. Am. Acad. Dermatol., 6:766–785, 1982).

However, due to highly toxic side effects of naturally occurring forms of vitamin A (hypervitaminosis A) at therapeutic dose level, clinical use of retinoids has been limited (Kamm et al., In: The Retinoids. Sporn et al., (eds.), Academic Press, N.Y., pp 228–326, 1984; Lippman et al.,

Cancer Treatment Reports, 71:493–515, 1987). In free form, the retinoids may have access to the surrounding normal tissues which might be the basis of their profound toxicity to liver, central nervous system, and skeletal tissue.

Therefore, one potential method to reduce the toxicity associated with retinoid administration would be the use of a drug delivery system. The liposomal format is a useful one for controlling the topography of drug distribution in vivo. This, in essence, involves attaining a high concentration and/or long duration of drug action at a target (e.g. a tumor) site where beneficial effects may occur, while maintaining a low concentration and/or reduced duration at other sites where adverse side effects may occur (Juliano, et al., In: Drug Delivery Systems, Juliano ed., Oxford Press, N.Y., pp 189–230, 1980). Liposome-encapsulation of drug may be expected to impact upon all the problems of controlled drug delivery since encapsulation radically alters the pharmacokinetics, distribution and metabolism of drugs.

There are additional difficulties in using a liposomal formulation of a retinoid for therapeutic purposes. For example, it is often desirable to store the composition in the form of a preliposomal powder, but many prior formulations are not satisfactory for such use, because they either contain an inadequate amount of retinoid, or they generate undesirable liposomes when they are reconstituted in aqueous solution.

For compositions that are to be administered intravenously, typically the composition must provide at least about 100 mg of the active ingredient in a single container; if it contains a lesser amount of the active ingredient, an impractically large number of vials will be needed for dosing a single patient.

Typically a vial having a volume of 120 cc is the largest that can be accommodated in a commercial freeze drier, and 50 cc is the maximum volume of liquid that can be filled in such a vial. If more than 1 g of lipids are included in 50 cc of liquid volume, the resulting liposomes after reconstitution have a size distribution which is not acceptable for parenteral administration. This is because the packing of the lipids during lyophilization is affected by the concentration of the lipids in the solution. Thus, the concentration of lipids in the solution must be limited. However, when this is done in previously-known liposomal retinoid formulations, the retinoid tends to crystallize, and separate from the liposomes shortly after reconstitution.

In order to both limit the concentration of lipids and supply a sufficient amount of retinoid, it is necessary to provide a molar ratio of retinoid to lipid greater than about 1 to 10. Previously known formulations have not had, and are believed not to be capable of having such a high packing of retinoid in the liposomes. Therefore, a need exists for improved compositions and methods which will minimize or eliminate the problems of the prior art.

SUMMARY OF THE INVENTION

The present invention relates to therapeutically useful, reduced toxicity compositions of carotenoids. The compositions comprise a carotenoid, lipid carrier particles, and an intercalation promoter agent. "Carotenoid" is used here to include retinoids, pro-retinoids, carotenes, xanthophylls, and analogs thereof. A preferred example is all-trans retinoic acid. The carotenoid is substantially uniformly distributed with the lipid in the lipid carrier particles. More particularly, the carotenoid is substantially uniformly distributed in an intercalated position throughout a hydrophobic portion of the lipid carrier particles, as opposed to the aqueous phase.

"Substantially uniformly distributed" means that at least 50% of the lipid carrier particles will contain carotenoid in a molar ratio between about 5:85 carotenoid:lipid and about 15:70. Preferably at least 75% of all lipid carrier particles will contain such a ratio of the active ingredient.

The composition is stable in an aqueous environment. In this context, "stable in an aqueous environment" means that the composition (1) will not exhibit any therapeutically significant degradation over a period of at least 24 hours, (2) will not exhibit a substantial degree of fusions of liposomes over that same period, and (3) will not exhibit substantial redistribution of the carotenoid over that same period, including no substantial movement of the drug into the aqueous phase of a liposome, and no substantial state change into a crystalline form.

The molar ratio of carotenoid to lipid in the lipid carrier particles is greater than about 1:10, and is most preferably at least about 15:85. The intercalation promoter agent preferably comprises at least about 15% by weight of the composition, and can suitably be, for example, a triglyceride.

"Lipid carrier particles" is used here to include liposomes, having a bilayer structure formed of one or more lipids having polar heads and nonpolar tails, as well as micelles, amorphous particulates of lipid, and other lipid emulsion state entities. When the particles are liposomes, suitable forms include multilamellar liposomes.

The present invention also relates to a pharmaceutical unit dosage formulation of a carotenoid, which comprises a carotenoid, lipid carrier particles, an intercalation promoter agent, and a pharmaceutically acceptable carrier. As stated above, the carotenoid is substantially uniformly distributed with the lipid in the lipid carrier particles, and the composition is stable in an aqueous environment.

In another aspect, the invention relates to a method of inhibiting the growth of cancer cells, in which a therapeutically effective amount of a carotenoid composition is administered to a living subject. The carotenoid composition can be as described above. The composition is preferably administered to the subject in a maintained molar ratio between about 5:85 carotenoid:lipid and about 15:70. "Maintained" in this context means that the stated ratio of drug to lipid lasts for at least 24 hours.

The present invention provides the therapeutic benefits of the carotenoid, while substantially reducing the undesirable toxicity of the composition, as compared to the free drug. For example, encapsulation of retinoic acid in liposomes results in a decrease of at least 15-fold in toxicity as compared to the free drug.

Further, the presence of the intercalation promoter agent permits the ratio of active ingredient to lipid to be increased above what has been previously known, and thus makes such formulations useful in a practical sense for lyophilization into a powder, and subsequent reconstitution into solution which can be administered parenterally to a patient. Without wishing to be bound by any particular theory, it is believed that the intercalation promoter agent overcomes steric hindrance that otherwise limits the amount of carotenoid that be incorporated in, for example, a liposome.

The encapsulation of carotenoids within, e.g., liposomes, permits their direct delivery to intracellular sites and thus circumvents the requirement for cell surface receptors. This may be of particular significance, for example, in therapy of tumors which lack the cell surface receptors for serum retinol binding protein but possess intracellular receptors for retinoic acid.

Compositions of the present invention are also substantially improved over prior liposomal retinoid formulations in terms of uniformity of drug distribution. Prior compositions often had substantial percentages of liposomes which contained essentially no drug. In the present invention, at least 50% and preferably at least 75% of all liposomes in the composition contain drug with the range specified above.

While not being bound by any particular theory of action, it has been found that, surprisingly, liposome encapsulation of carotenoids and particularly all-trans retinoic acid, circumvents the usual hepatic clearance mechanisms. This has resulted in a substantial extension of the efficacy of liposomal carotenoid over free carotenoid or retinoid. It is believed that liposomal all-trans retinoic acid avoids the problems of resistance to non-liposomal all-trans retinoic acid. This resistance is displayed by such parameters as reduced serum concentration upon prolonged treatment typically observed in treatments as extended over 2, 5 or 7 weeks or longer. Here, substantially longer periods of drug administration were unaccompanied by reduced circulating drug levels. Therapeutic i.v. dosages of 15 mg/m^2, 30 mg/m^2, 60 mg/m^2, 75 mg/m^2, and 90 mg/m^2, and further including 150 mg/m^2, 300 mg/m^2 and higher are noted. Regimens of therapy extending in excess of 7 weeks, and further in excess of 14 weeks, and further exhibiting non-declining drug levels are particularly noted. Regimens of administration of all-trans retinoic acid that avoid retinoid resistance are particularly noted herein, which includes administration of liposomal all-trans retinoic acid, and in one embodiment includes the retinoid being intercalated in the liposomal bilayer in substantially uncrystallized form.

In vivo administration of liposomal all-trans retinoic acid over a prolonged period did not exhibit declining blood levels. In vitro studies of isolated liver microsomes revealed unchanged catabolism upon repeated exposure to liposomal all-trans retinoic acid. In contrast, microsomes isolated from subjects originally administered non-liposomal all-trans retinoic acid an equal number of times displayed increased metabolism of all-trans retinoic acid.

Test data has indicated that the instant liposomal carotenoid formulations avoid "retinoid" resistance upon chronic i.v. administration. The results suggest that chronic administration of liposomal carotenoid, and particularly all-trans retinoic acid, does not affect the levels of circulating drug in subjects. While phospholipids are preferred, the broad grouping of lipids are useful in forming particular liposomes. Liver microsomes from test animals did not show any significant change in their ability to metabolize all-trans retinoic acid. In contrast, long-term oral administration of non-liposomal all-trans retinoic acid caused a significant decrease in circulating drug levels after 7 weeks of treatment. Liver microsomes from these animals converted all-trans retinoic acid into polar products much more rapidly than microsomes obtained from liposomal all-trans retinoic acid-treated or untreated animals.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a time profile of liposomal retinoic acid (L-RA) stability in the presence (●) and absence (○) of serum.

FIG. 2 shows human red blood cell (RBC) lysis as a function of time with RA (●) and L-RA (▲).

FIG. 3 shows RBC lysis as a function of retinoic acid (RA) concentration (●) and L-RA concentration (▲).

FIG. 4 shows the inhibition of THP-1 cell growth as a function of RA concentration (●), L-RA concentration (○) or empty liposome concentration (Δ).

FIG. 5 shows the induction of transglutaminase (TGase) in human monocytic THP-1 cells as a function of treatment with RA or L-RA.

FIG. 6 shows the inhibition of human histiocytic U-937 cell growth as a function of RA concentration (●), L-RA concentration (○) and empty liposome concentration (Δ).

FIG. 7 shows the time course of accumulation of tissue TGase activity in cultured human peripheral blood monocytes (HPBM). HPBM were fractionated into small (○) and large (●) subpopulations by centrifugal elutriation, and they were cultured in 35-mm-well tissue culture plates as described in Materials and Methods. At the indicated time points the cells were washed, sonicated, and assayed for TGase activity. Values are the means of six determinations from two dishes.

FIG. 8 shows dose-dependent effects of recombinant interferon-gamma (rIFN-g) on induction of tissue TGase activity in HPBM subpopulations. Small (○) and large (●) monocytes were cultured in serum containing medium alone or medium containing increasing concentrations of rIFN-g. After 72 hr, the cells were harvested and the cell lysates assayed for tissue TGase activity. The results shown represent mean ±SD of three determinations from an individual donor.

FIGS. 9A and B show effects of retinol (ROH) and RA on induction of tissue TGase activity in cultured HPBM. Cells were cultured in the presence of 5% human AB serum and the absence (●) or presence of 500 nM ROH (▲) or RA (○) for varying periods of time. At the end of each time point, the cells were harvested and assayed for enzyme activity. Values shown are the means ±SD of six determinations from two independent experiments. Inset, dose-response curve for tissue TGase induction by ROH (▲) and RA (●) in HPBM after 72-hr culture.

FIGS. 10A and B show effects of free- and liposome-encapsulated RA on induction of tissue TGase in HPBM. A: The cells were cultured in tissue culture dishes in presence of serum-containing medium alone (Δ) 500 nm liposomal RA (●), or medium containing 500 nM free-RA (▲), or "empty liposomes" (○) for indicated periods of time. Both the liposomal RA and "empty liposomes" contained 200 μg/ml lipid. At the end of each time point, the cultures were washed and cell lysates assayed for TGase activity. Values shown are the mean ±SD of six determinations from two independent experiments. B: Western-blot analysis of the levels of tissue TGase in freshly isolated HPBM (lane 1) and in HPBM cultured for 72 hr in the presence of serum-containing medium alone (lane 2), in medium containing 500 nM free RA (lane 3), 500 nM liposomal RA (lane 4), or "empty liposomes" (lane 5). Cell lysates containing 25 ug of protein were subjected to Western-blot analysis as described in Materials and Methods.

FIGS. 11A and B show effect of free and liposome-encapsulated ROH on induction of tissue TGase in HPBM. A: HPBM monolayers were cultured in serum-containing medium alone (Δ) or medium containing 1 μM of free- (○) or liposomal-ROH (▲) for 72 hr. Then the cultures were washed and the cell lysates assayed for enzyme activity as described in Materials and Methods. B: Western-blot analysis of tissue TGase levels in freshly isolated HPBM (lane 1) and in HPBM cultured for 72 hr in the presence of serum-containing medium alone (lane 2), in medium containing 1 μM of free ROH (lane 3), or liposome-encapsulated ROH (lane 4) as described in Materials and Methods. Twenty-five micrograms of cell protein was loaded onto each lane.

FIG. 12(A) shows the levels of all-trans retinoic acid in the blood 60 min after oral administration of non-liposomal all-trans retinoic acid or i.v. administration of liposomal all-trans retinoic acid.

FIG. 12 (B) shows blood clearance of all-trans retinoic acid following administration of the last dose of all-trans retinoic acid.

FIG. 13(A) shows the percentage of all-trans retinoic acid metabolized by isolated liver microsomes to animals exposed to 7 weeks of treatment with all-trans retinoic acid, either liposomal i.v. or oral.

FIG. 13(B) shows radioactivity (cpm) of all-trans retinoic acid or its polar metabolites (as discussed in association with FIG. 12(A)) as gathered from five animals.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

Suitable therapeutic carotenoids for encapsulation in accordance with the present invention include various retinoids. Trans-retinoic acid and all-trans-retinol are preferred. Other retinoids that are believed suitable include: retinoic acid methyl ester, retinoic acid ethyl ester, phenyl analog of retinoic acid, etretinate, retinol, retinyl acetate, retinaldehyde, all-trans-retinoic acid, and 13-cis-retinoic acid.

Lipid carrier particles, such as liposomes, can be formed by methods that are well known in this field. Suitable phospholipid compounds include phosphatidyl choline, phosphatidic acid, phosphatidyl serine, sphingolipids, sphingomyelin, cardiolipin, glycolipids, gangliosides, cerebrosides, phosphatides, sterols, and the like. More particularly, the phospholipids which can be used include dimyristoyl phosphatidyl choline, egg phosphatidyl choline, dilauryloyl phosphatidyl choline, dipalmitoyl phosphatidyl choline, distearoyl phosphatidyl choline, 1-myristoyl-2-palmitoyl phosphatidyl choline, 1-palmitoyl-2-myristoyl phosphatidyl choline, 1-palmitoyl-2-stearoyl phosphatidyl choline, 1-stearoyl-2-palmitoyl phosphatidyl choline, dioleoyl phosphatidyl choline, dimyristoyl phosphatidic acid, dipalmitoyl phosphatidic acid, dimyristoyl phosphatidyl ethanolamine, dipalmitoyl phosphatidyl ethanolamine, dimyristoyl phosphatidyl serine, dipalmitoyl phosphatidyl serine, brain phosphatidyl serine, brain sphingomyelin, dipalmitoyl sphingomyelin, and distearoyl sphingomyelin.

Phosphatidyl glycerol, more particularly dimyristoyl phosphatidyl glycerol (DMPG), is not preferred for use in the present invention. In the carotenoid compositions of the present invention, the presence of DMPG correlates with the appearance of amorphous structures of anomalous size, which are believed to render the composition much less suitable for intravenous administration. When DMPG is omitted, the amorphous structures are not observed. The undesirable effects that are apparently caused by the presence of DMPG may result from the fact that DMPG has a negative charge, which may interact with the carboxylate of the carotenoid.

In addition, other lipids such as steroids and cholesterol may be intermixed with the phospholipid components to confer certain desired and known properties on the resultant liposomes. Further, synthetic phospholipids containing either altered aliphatic portions, such as hydroxyl groups, branched carbon chains, cyclo derivatives, aromatic derivatives, ethers, amides, polyunsaturated derivatives, halogenated derivatives, or altered hydrophilic portions containing carbohydrate, glycol, phosphate, phosphonate, quaternary amine, sulfate, sulfonate, carboxy, amine, sulfhydryl, imidazole groups and combinations of such groups, can be either substituted or intermixed with the phospholipids, and others known to those skilled in the art.

A suitable intercalation promoter agent will permit the high molar ratio of carotenoid to lipid that is desired for the present invention, without substantial crystallization from the liposomes after they are reconstituted in aqueous solution, as can be observed by microscopic analysis, separation techniques based on buoyant density, or other techniques well known to those skilled in the art. Triglycerides are preferred intercalation promoter agents, with soybean oil as one specific example. Other suitable agents include sterols, such as cholesterol, fatty alcohols, fatty acids, fatty acids esterified to a number of moieties, such as polysorbate, propylene glycol, mono- and diglycerides, and polymers such as polyvinyl alcohols.

Prior to lyophilization, the carotenoid, lipids, and intercalation promoter agent can be dissolved in an organic solvent, such as t-butanol. Lyophilization to form a preliposomal powder can be performed using commercial apparatus which is known to persons skilled in this field. After lyophilization, the powder can be reconstituted as, e.g., liposomes, by adding a pharmaceutically acceptable carrier, such as sterile water, saline solution, or dextrose solution, with agitation, and optionally with the application of heat.

A preferred formulation, which can be dissolved in 45 ml of t-butanol, is as follows:

component	mg	millimoles	mole %	wt %
DMPC	850	1.28	72	77
soybean oil	150	0.17	9	14
tretinoin	100	0.33	19	9

A composition of the present invention is preferably administered to a patient parenterally, for example by intravenous, intraarterial, intramuscular, intralymphatic, intraperitoneal, subcutaneous, intrapleural, or intrathecal injection. Administration could also be by topical application or oral dosage. Preferred dosages are between 40–200 mg/m^2. The dosage is preferably repeated on a timed schedule until tumor regression or disappearance has been achieved, and may be in conjunction with other forms of tumor therapy such as surgery, radiation, or chemotherapy with other agents.

The present invention is useful in the treatment of cancer, including the following specific examples: hematologic malignancies such as leukemia and lymphoma, carcinomas such as breast, lung, and colon, and sarcomas such as Kaposi's sarcoma.

EXAMPLE 1

Preparation of liposomal-all trans-retinoic acid (L-RA)

Preparation of lyophilized powder containing all trans-retinoic acid and phospholipids was carried out as follows. A solution of retinoic acid in t-butanol (1–5 mg/ml) was added to a dry lipid film containing dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) at a 7:3 molar ratio. The phospholipids were solubilized in the t-butanol containing the all-trans retinoic acid and the solution was freeze-dried overnight. A powder containing dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), and all-trans retinoic acid was obtained. The lipid:drug ratio used was from 10:1 to 15:1.

Reconstitution of liposomal retinoic acid from the lyophilized powder was done as follows. The lyophilized powder was mixed with normal saline at room temperature to form multilamellar liposomes containing all trans-retinoic acid. This reconstitution method required mild hand-shaking for 1 min to obtain a preparation devoid of any aggregates or clumps. By light microscopy, the reconstituted preparation contained multilamellar liposomes of a close size range. No aggregates or drug clumps were identified in the liposomal preparation in three different experiments.

Encapsulation efficiency and size distribution of the liposomal all-trans retinoic acid preparation were determined as follows. The liposomal all-trans retinoic acid preparation was centrifuged at 30,000×g for 45 minutes. A yellowish pellet containing the retinoic acid and the lipids was obtained. By light microscopy, the pellet was composed of liposomes with no crystals or drug aggregates. The encapsulation efficiency was calculated to be greater than 90% by measuring the amount of free retinoic acid in the supernatant by UV. spectrophotometry. Liposomes were sized in a Coulter-Counter and Channelizer. The size distribution was as follows: 27% of liposomes less than 2 micrometers (μm), 65% between 2 μm and 3 μm, 14% between 3 μm and 5 μm, 1% more than 5 μm. The method used for encapsulation of retinoids was simple, reproducible and could be used for large scale production, for example, for clinical trials.

Further experiments were performed by the same procedure but with different lipids, ratios of lipids and the use of ^{3}H-all-trans retinoic acid. Additional lipids utilized were dipalmitoyl phosphatidyl choline (DPPC) stearylamine (SA) and cholesterol. After sedimentation of the liposomes, residual ^{3}H was determined and encapsulation efficiency calculated. Table 1 shows encapsulation efficiencies determined by this method for various L-RA preparations.

TABLE 1

Encapsulation Efficiency of Retinoic Acid in Liposomes

LIPOSOME COMPOSITION	ENCAPSULATION EFFICIENCY (%)
DMPC:cholesterol 9:1	69.3
DMPC:cholesterol 9:3	64.5
DPPC	69.1
DMPC:SA:cholesterol 8:1:1	56.7
DMPC:DMPG 7:3	90
DMPC:DMPG 9:1	90.7

Of the lipid compositions studied, DMPC:DMPG at ratios between 7:3 and 9:1 gave superior encapsulation efficiencies. Liposomal all-trans retinol (L-ROH) was prepared by the methods described above for L-RA with DMPC:DMPG, 7:3.

EXAMPLE 2

Stability of Liposomal Retinoic Acid

Liposomal ^{3}H-retinoic acid (L-^{3}H-RA) was prepared with DMPC:DMPG, 7:3 as described in Example 1. Samples of the L-^{3}H-RA were incubated with either phosphate-buffered saline (PBS) or PBS with 20% (by volume) fetal calf serum (FCS). After various periods of incubation at about 37° C., aliquots were removed and centrifuged to sediment liposomes. The tritium in the supernatant solution was measured to determine ^{3}H-RA release. FIG. 1 shows the release of ^{3}H-RA over a two day period. The L-^{3}H-RA was over about 80% stable over the period of the experiment, even in the presence of 20% FCS.

When ^{3}H-all-trans retinol was used to label L-ROH and stability in PBS measured, only about 5% of the ^{3}H-ROH was released after a 24 hr incubation at 37° C.

EXAMPLE 3

In Vitro Lysis of Human Erythrocytes (RBCS) by Retinoic Acid or Liposomal Retinoic Acid

Lysis of human red blood cells (RBCs) was quantitated by measuring the release of hemoglobin in the supernatants by observation of increases in optical density at 550 nanometers (nm), as described previously (Mehta, et al., Biochem. Biophys, Acta., Vol. 770-, pp 230–234 (1984). Free-RA dissolved in dimethyl formamide (DMFA), was added to the RBCs. Results with appropriate solvent controls, empty liposomes, and empty liposomes plus free-drug were also noted. Release of hemoglobin by hypotonic lysis of the same number of human RBCs by water was taken as a 100% positive control, while cells treated with PBS were taken as negative controls.

Preparations of L-RA comprising various lipids were incubated at a concentration of 20 microgram (μg) RA per ml with RBCs in PBS for 4 hr at 37° C. The toxicity of the L-RA preparations on the basis of percent RBC lysis is shown in Table 2.

TABLE 2

In Vitro Toxicity Of L-RA Preparations To RBCs

LIPOSOME COMPOSITION	% RBC LYSIS
DMPC:Cholesterol 9:1	4.5
DMPC:Cholesterol 9:3	90.2
DPPC	6.7
DMPC:SA:Cholesterol 8:1:1	70.4
DMPC:DMPG 7:3	8
DMPC:DMPG 9:1	8.3

As may be seen from the data of Table 2, L-RA of DMPC:cholesterol, DPPC, DMPC:DMPG (7:3) and DMPC:DMPG (9:1) exhibited low RBC toxicity under these conditions. It is of interest to note that the latter two L-RA compositions exhibited superior encapsulation efficiencies (Table 1).

A further experiment concerning the toxicity over time of free RA and L-RA (DMPC:DMPG-7:3) toward RBC was conducted. Human erythrocytes were incubated at 37° C. in PBS with 10 ug/ml free RA or 120 ug/ml L-RA, and RBC lysis monitored over a period of 5 hr. FIG. 2 shows time courses of RBC lysis. At between about 1 hr and about 3 hr, the free RA extensively lysed a large majority of the erythrocytes. When a similar manipulation was performed with L-RA (DMPC:DMPG(7:3)) at a RA concentration of 120 ug/ml, little RBC lysis occurred (e., less than 10% after 6 hr).

A study was also conducted concerning the effects upon RBC lysis in 2 hr of free RA and L-RA (DMPC:DMPG(7:3)) at various concentrations. FIG. 3 shows the results of this study. Free RA showed linearly increasing RBC lysis between about 5 ug RA/ml and about 30 ug RA/ml. Liposomal RA caused RBC lysis of only about 5% at a concentration of 160 ug RA/ml.

EXAMPLE 4

Acute Toxicity of Free and Liposomal Retinoic Acid

The acute toxicity of free and liposomal all-trans retinoic acid was studied in CD1 mice. Free all-trans retinoic acid was prepared as an emulsion in normal saline containing 10% DMSO and 2% Tween 80 at a concentration of 3 to 5 mg/ml. Liposomal all-trans retinoic acid was prepared using a lipid:drug ratio of 15:1. The final concentration of all-trans retinoic acid in the liposomal preparation was 3 mg/ml. Empty liposomes of the same lipid composition (DMPC:DMPG 7:3) were also tested at doses equivalent to 80 mg/kg, 100 mg/kg, and 120 mg/kg of liposomal-all trans retinoic acid. Normal saline containing 10% DMSO and 2% Tween 80 was also tested as a control at a dose equivalent to 50 mg/kg of free all-trans retinoic acid. All drugs tested were injected intravenously via tail vein as a single bolus. The injected volumes of free and liposomal-all-trans retinoic acid were the same for each dose.

Table 3 shows data obtained from these acute toxicity experiments.

TABLE 3

Acute Toxicity of Free and Liposomal All-Trans Retinoic Acid

Drug	Dose (mg/kg)	Number Animals with seizures	Number Animals alive (72 hr)
Free RA	10	0/6	6/6
	20	6/6	5/6
	30	6/6	4/6
	40	3/3	0/3
	50	3/3	0/3
L-RA	40	0/6	6/6
	60	0/6	6/6
	80	0/6	6/6
	100	0/6	6/6
	120	0/6	6/6
Empty Liposomes	80	0/6	6/6
	100	0/6	5/6
	120	0/6	6/6
Normal saline 10% DMSO 2% Tween 80	50	0/6	6/6

The maximum non-toxic dose of free all-trans retinoic acid was 10 mg/kg. Higher doses caused seizures immediately after injection. The acute LD_{50} (deaths occurring up to 72 hours after injection) of free all-trans retinoic acid was 32 mg/kg. The cause of death was cardiopulmonary arrest after seizures for 1–2 minutes in all animals. No seizures or deaths were observed in the animals treated with liposomal all-trans retinoic acid at a dose of 120 mg/kg (maximum non-toxic dose and LD_{50} greater than 120 mg/kg). Higher doses were not tested. No seizures were observed in the animals treated with empty liposomes or normal saline with 10% DMSO and 2% Tween 80.

EXAMPLE 5

In Vitro Inhibition of Tumor Cell Growth

Liposomal all-trans retinoic acid (L-RA) was prepared as described in Example 1.

Cells of the human monocytic cell line THP-1 were inoculated into samples of eucaryotic cell culture medium in the presence or absence of L-RA, at a final RA concentration of 1 micromolar (μm). After 24 hr at 37° C., ^{3}H-thymidine was added to each culture and incorporation thereof into cellular polynucleotides measured. Table 4 shows the percentage of tumor growth inhibition as reflected by decreases in ^{3}H-thymidine incorporation induced by L-RA of differing lipid compositions.

TABLE 4

L-RA Inhibition of Tumor Cell Growth

LIPOSOME COMPOSITION	TUMOR CELL (THP-1) INHIBITION (%)
DMPC:Cholesterol 9:1	72
DMPC:Cholesterol 9:3	22
DPPC	8
DMPC:SA:Cholesterol 8:1:1	84
DMPC:DMPG 7:3	70
DMPC:DMPG 9:1	32

From Table 4, it should be noted that L-RA (DMPC:DMPG-7:3), which, as previously shown herein, gave a superior encapsulation efficiency and showed-a low RBC toxicity (Tables 1 and 2), also effectively inhibited the tumor cell growth.

Cells of the human monocytic cell line THP-1 and of the human histiocytic cell line U-937 were inoculated at about 20,000 cells per cell in aliquots of eucaryotic cell culture medium contained in wells of a 96 well microtiter plate. The medium in various wells contained different amount of free RA or L-RA (DMPC:DMPG 7:3). The cells were incubated for 72 hr at 37° C. and cell growth determined and compared to that of controls without any form of retinoic acid. FIG. 4 shows the inhibition of THP-1 cell growth by increasing concentrations of free RA or L-RA (DMPC:DMPG 7:3). At concentrations of less than 1 μg RA/ml, both preparations inhibited cell growth by over 90%.

The human monocytic leukemia THP-1 cells, after a 72 hr incubation with either free RA or L-RA at a concentration of 0.3 μg RA/ml, were observed to have lost their generally ovate form and to have a more flattened and spread morphological appearance often associated with cellular differentiation. The generally ovate form was retained when the cells were cultured in the absence of any free or liposomal retinoic acid.

After incubation for 24 hr with 0.3 μg/ml or 0.6 μg/ml RA or L-RA in another experiment, THP-1 cells had increased levels of tissue transglutaminase enzymic activity, a marker for monocytic cell differentiation. As shown in FIG. 5, THP-1 cells, at 4×10^6 cells/ml, showed about 500% greater transglutaminase activity when incubated with L-RA as compared to free RA at equivalent retinoic acid concentrations.

Cells of the human histiocytic cell line U-937 were distributed and cultured under the same conditions as the THP-1 cells in the prior experiment. FIG. 6 shows the effects upon cell growth of increasing concentrations of free all-trans retinoic acid (RA), liposomal (DMPC:DMPG 7:3) all-trans retinoic acid (L-RA) and empty liposomes (which were devoid of retinoic acid). It should be noted that the U-937 cells were almost completely growth-inhibited by L-RA at a retinoic acid concentration of about 10 ug/ml while this amount of free RA inhibited growth less than 50%.

EXAMPLE 6

Antitumor Activity of Liposomal All-Trans Retinoic Acid in vivo

The antitumor activity of liposomal-all trans retinoic acid (DMPC:DMPG 7:3) was tested in vivo against liver metastases of M5076 reticulosarcoma. C57BL/6 mice were inoculated with 20,000 M5076 cells on day 0. Intravenous treatment with 60 mg/kg liposomal all-trans retinoic acid was given on day 4. The mean survival of control animals (non-treated) was 21.8+1.6 days. The mean survival of treated animals was 27.0±1.6 days. Liposomal all-trans retinoic acid was shown, therefore, to have antitumor activity at a dose well below the maximum non-toxic dose, against a cell line (M5076) which was resistant to free retinoic acid in in vitro studies. THP-1 cells treated in vitro with RA (1 MM) for 72 hours when injected subcutaneously into male mice, failed to develop into tumors, whereas untreated cells formed a huge mass of tumors in such mice.

EXAMPLE 7

Induction of Tissue Transglutaminase in Human Peripheral Blood Monocytes by Intracellular Delivery of Retinoids

Circulating blood monocytes are the precursors of macrophages which accumulate at the sites of tumor rejection [2], delayed hypersensitivity [25], chronic inflammation [6], and at the site of damaged tissue as a part of the healing processes [11] (see reference citations in section D). At these sites, peripheral blood monocytes acquire new functional and biochemical characteristics that are associated with the maturation or differentiation process. To understand clearly the mechanisms involved in differentiation, it is necessary to manipulate the extracellular environment and assess precisely a variety of cellular functions and biochemical activities.

Vitamin A and its analogues (retinoids) have been shown to exert a profound effect on the differentiation of monocytic cells. Both normal [19] and leukemic [7,17,28] monocytic cells differentiate in response to retinoids which might suggest that retinoids play a role in regulating the differentiation of these cells. According to recent reports, the cellular activity of transglutaminase (TGase), an enzyme that catalyzes the covalent cross-linking of proteins, may be directly linked to the retinoid's action [4,15,21,23,35,39,39]. Recently, the present inventors found that in vitro maturation of human peripheral blood monocytes (HPBM) to macrophage-like cells was associated with the induction and accumulation of a specific intracellular TGase, tissue TGase [19,22]. Gamma (g)-interferon, which promotes the tumoricidal properties in HPBM, also augmented the expression of tissue TGase [19]. Similarly, the activation of guinea pig and mouse macrophages in vivo was associated with a marked increase in tissue TGase activity [10,24,34]. Terminal differentiation of human monocytic leukemia cells (THP-1) induced by phorbol ester and retinoic acid was associated with induction and accumulation of tissue TGase (17], suggesting that the induction of tissue TGase was a marker of monocytic cell differentiation. The present invention involves further definition of the role of retinoids in differentiation and maturation of HPBM and comprises studies of culture conditions that inhibit or facilitate the internalization of retinoids by HPBM on expression of tissue TGase. The studies herein demonstrate that HPBM, isolated into two subpopulations, show no significant difference in their ability to express tissue TGase activity induced by either in vitro culture or exposure to recombinant interferon gamma (rIFN-g), and that the expression of tissue TGase in cultured HPBM may be induced by a direct delivery of retinoids to intracellular sites.

A. Materials and Methods

1. Materials

RPMI-1640 medium supplemented with L-glutamine and human AB serum were from Gibco Laboratories (Grand Island, N.Y.); *Escherichia coli*-derived human recombinant g-interferon (rIFN-g) was kindly supplied by Genentech Inc. (South San Francisco, Calif.); and all-trans retinol (ROH) and all-trans retinoic acid (RA) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The chromatographically pure lipids dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) were from Avanti Polar Lipids (Birmingham, Ala.); tritiated putrescine (sp. act. 28.8 Ci/mmol), from New England Nuclear (Boston, Mass.); and tritiated ROH (sp. act. 15 mci/mmol), from Amersham (Arlington Heights, Ill.). Lipids, culture medium, and serum were screened for endotoxin with the Limulus amebocyte lysate assay (MA Bioproducts, Walkersville, Md.), and they were used only when endotoxin contamination was less than 0.25 ng/ml.

2. HPBM Isolation, Purification, and Culture

Pure populations of HPBM were obtained by countercurrent centrifugal elutriation of mononuclear leukocyte-rich fractions obtained from normal donors who were undergoing routine plateletpheresis [12]. HPBM were isolated into two subpopulations according to size with a Coulter ZBI counter and C-1000 channelizer (Coulter Electronics, Hialeah, Fla.). The median volume of small monocytes was 255 mm^3, and that of the large monocytes was 280 mm^3. The small monocytes were 95%±3% nonspecific esterase-positive and the large monocytes were 98%±2% positive. Detailed procedures for isolation and characteristics of these subpopulations have been published elsewhere [36,37]. Small, large, or mixed (obtained by mixing equal parts of small and large HPBM) HPBM subpopulations were washed once with medium (RPMI-1640 supplemented with L-glutamine, 20 mM HEPES buffer, 20 ug/ml gentamicin, and 5% human AB Serum) and resuspended to 0.5 million/ml density in the same medium. The cells were dispensed in 4-ml samples into 35-mm-well plates and cultured under appropriate conditions.

3. Enzyme Assay

Tissue TGase activity in cell extracts was measured as a Ca^{2+}, dependent incorporation of [^{3}H] putrescine into dimethylcasein. In brief, cultured HPBM were washed three times in Tris-buffered saline (20 mM Tris-HCl, 0.15M NaCl, pH 7.6) and scraped from the dish in a minimal volume of the same buffer containing 1 mM EDTA and 15 mM Beta-mercaptoethanol. The cells were lysed by sonication, and TGase activity in the lysates was determined as described previously [13,20]. The protein content in cell lysates was determined by Lowry's method [14] with bovine gamma globulin as standard. The enzyme activity was expressed as nanomoles of putrescine incorporated into dimethyl-casein per hour per milligram of cell protein.

4. Immunochemical Detection of Tissue TGase

To detect tissue TGase in cell extracts, the cell lysates were solubilized in 20 mm Tris-HCl (pH 6.8) containing 1% sodium dodecyl sulfate (SDS), 0.75M Beta-mercaptoethanol, 2.5% sucrose and 0.001% bromophenol blue. Solubilized extracts were fractionated by electrophoresis on a 6.5% discontinuous polyacrylamide gel and electroblotted onto nitrocellulose paper. The paper was neutralized with 5% bovine serum albumin and treated with iodinated anti-tissue TGase antibody; the preparation, characterization and properties of this antibody have been described elsewhere (24]. The unbound antibody was removed by washing the paper in Tris-HCl buffer (50 mM, pH 7.5) containing 200 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 0.25% gelatin, and the paper was dried and autoradiographed as described earlier [20,24].

5. Preparation of Liposomes

Multilamellar vesicles (liposomes) containing DMPC and DMPG at a molar ratio of 7:3 were prepared as described [16,18]. All-trans ROH or RA were encapsulated by adding the required amount of the drug (predissolved in ethanol) in lipid-containing organic solvents before vacuum drying. The dried lipid-drug film was dispersed by agitation in sterile saline solution. Retinoids up to a 1:10 drug:lipid ratio could be completely encapsulated within the liposomes and were highly stable. The stability and encapsulation efficiency of the liposome preparations were studied by using radiolabeled retinol and showed that only 5%±2% of the incorporated radioactivity leaked out in the supernatant after 24-hr incubation at 37° C.

6. Binding Assay for [^{3}H]ROH

Freshly isolated HPBM were cultured in serum containing medium alone or medium plus 50 units (U)/ml rIFN-g for varying periods of time. At the end of indicated time periods, HPBM monolayers were washed twice in ice cold medium and resuspended in 0.5 ml of prechilled reaction mixture containing 5.0 microcuries (μCi)/ml [11,12(n)^{3}H] vitamin A (free ROH) in RPMI medium supplemented with 5% delipidized human AB serum (serum delipidization was done by organic solvent extraction as described earlier [33]. Binding assays were carried out for 1 hr in an ice bath. After a 1-hr incubation, the monocyte monolayers were washed six times with ice-cold medium and the cells were lysed in 200 μl of Triton X-100. Fifty-microliter aliquots of cell lysates, in triplicate, were counted for the cell-associated radioactivity. Background counts, obtained by adding the reaction mixture toward the end of the 1-hr incubation before harvesting, were subtracted from the experimental values.

B. Results

1. Tissue TGase Induction During In Vitro Culture of HPBM

The culture of HPBM in the presence of serum-containing medium for up to 10 days was associated with a marked induction of tissue TGase activity in both small and large HPBM (FIG. 7), the increase in enzyme activity being more rapid after about 4 days of culture. After 10 days in culture, small monocytes showed a 93-fold increase in enzyme activity (from 0.44 to 41.1 nmol/hr/mg), whereas large HPBM accumulated about 103-fold increase in the enzyme activity (from 0.36 to 37.4 nmol/hr/mg). Small and large HPBM mixed together and cultured under similar conditions showed no significant difference in the rate and amount of accumulation of tissue TGase activity compared with that of individual HPBM fractions (data not shown). Induction of enzyme activity was associated with a change in the morphology of cultured monocytes. Freshly isolated HPBM looked rounded, but after 6–8 days in culture both the large and small HPBM became firmly adherent to the plastic surface, were more spread and flattened, and had the appearance typical of mature macrophages. By day 10, when the cells had accumulated maximal levels of enzyme activity, these levels then either plateaued or started declining.

2. Effect of rIFN-g on Tissue TGase Expression

The effect of continuous exposure to rIFN-g on induction of tissue TGase activity in HPBM is shown in FIG. 8. Small and large monocytes were cultured in serum-containing medium for 72 hr in the presence of increasing concentrations of rIFN-g. Enzyme activity in the HPBM populations increased significantly after their continuous exposure to rIFN-g compared with that of cells cultured in the presence

of medium along. However, rIFN-g dose size produced no significant difference in enzyme activity between the two HPBM populations. As previously noted [19], a 100-U/ml dose of rIFN-g seemed to be optimal for augmenting TGase activity; higher rIFN-g-concentrations were less effective. The inductive effect of rIFN-g on tissue TGase activity was evidence at 5 U/ml and pretreatment of HPBM cultures with rIFN-g (100 U/ml) followed by washing and subsequent culture in medium alone did not enhance the expression of tissue TGase. The rIFN-g-induced augmentation of tissue TGase was associated with morphologic changes in HPBM so that the rIFN-g-treated cells were more spread out and flattened than the untreated control cells after three days in culture.

3. Effect of Retinoids on Tissue TGase Induction

Since the two HPBM populations showed no heterogeneity in terms of induced tissue TGase levels, our subsequent studies were done with whole HPBM fraction without separation into subsets. HPBM cultured in the presence of 500 nM RA for 24 hr accumulated at least three-fold higher enzyme activity than did the control cells cultured in medium along (FIG. 9). Continuous exposure to RA caused a rapid and linear increase in the enzyme activity, whereas in the control cells no significant change in the level of tissue TGase activity was observed for up to 2 days of culture. By day 3, the control cells accumulated about six-fold higher enzyme activity (3.4 nmol/hr/mg) than did freshly isolated HPBM (0.6 nmol/hr/mg), but they still had significantly less enzyme activity than the RA-treated cells (9.8 nmol/hr/mg). Retinoic acid-induced expression of tissue TGase was dose dependent (FIG. 9 inset). ROH, the physiologic analogue of RA, did not induce the expression of tissue TGase in HPBM even at a dose of 1 μM. Thus, HPBM cultured in the presence of ROH for up to 3 days showed no significant difference in accumulation of tissue TGase activity when compared with that of control cells cultured in medium along (FIG. 9).

4. Effect of Liposome-Encapsulated Retinoids on Tissue TGase Induction

Liposome-encapsulated RA was more effective in inducing tissue TGase expression than was free RA at an equimolar concentration. After 24-hr culture, the amount of tissue TGase activity in HPBM induced by free or liposomal RA at an equimolar concentration of 500 nM was not significantly different (3.4 and 3.7 nmol/hr/mg, respectively); after 48 and 72 hr, however, liposomal RA-treated cells accumulated at least 50% more enzyme activity than did free RA-treated cells (FIG. 10A). That increase in enzyme activity by liposome-encapsulated RA was a specific effect of RA and not of lipids was demonstrated by the fact that a culture of HPBM in the presence of "empty liposomes," and containing equivalent amount of lipids did not induce enzyme activity throughout the incubation period. "Empty liposomes," as reported earlier [20], inhibited serum-induced expression of tissue TGase after 72 hr of culture (FIG. 10A). The free or liposomal RA-induced increase in enzyme activity was caused by an increased amount of the enzyme peptide, as revealed by Western-blot analysis of cell lysates using a iodinated antibody to tissue TGase (FIG. 10B). The increase in enzyme activity was proportional to the increase in enzyme peptide and not caused by activation of preexisting enzyme.

Retinol, which in its free form was unable to enhance the expression of tissue TGase in HPBM, became active when presented in liposomal form. Liposome-encapsulated ROH caused a rapid and linear increase in tissue TGase activity with time in culture (FIG. 11A). After 72 hr of culture,

liposomal-ROH caused a nine-fold increase in enzyme activity (7.1 nmol/hr/mg) when compared to that of control cells exposed to free ROH under similar conditions (0.8 nmol/hr/mg). Liposomal ROH-induced expression of tissue TGase resulted from increased accumulation of the enzyme peptide as demonstrated by Western-blot analysis (FIG. 11B).

5. Tissue TGase induction is Related to HPBM Uptake of Retinoids

The effect of in vitro maturation and rIFN-g treatment on the binding of tritiated-ROH by HPBM was examined. After 4 days of control culture (medium dose), tritiated-ROH binding by HPBM increased 50% compared to this binding by freshly isolated cells. After 9 days the control culture binding value increased to 350%. The increases in ROH binding were associated with parallel increases in tissue TGase activity (Table 5).

TABLE 5

Effect of In Vitro Culture and rIFN-g Treatment on [^{3}H] ROH Binding by HPBM

Culture Conditions	Days in Culture	[^{3}H] ROH bound (cpm/10 μg protein)	Tissue TGase activity (nmol/hr/mg)
medium alone	0	684 ± 25	0.25 ± 0.13
	4	994 ± 115	2.96 ± 0.75
	9	2,220 ± 144	32.60 ± 8.50
medium alone	3	626 ± 37	2.9 ± 0.23
medium + rIFN-g	3	1,782 ± 130	7.6 ± 0.7

[a]HPBM were cultured in serum-containing medium alone or medium containing 50 U/ml rIFN-g for indicated periods of time.
[b]Binding of tritiated ROH during different periods of culture was determined as described in Materials and Methods.
[c]Parallel cultures of HPBM maintained under similar conditions were used for assaying enzyme activity as described in Materials and Methods.

Exposure of HPBM to rIFN-g augmented the ROH binding and the expression of enzyme activity. The rIFN-g-treated cells showed a threefold higher [^{3}H]ROH binding than did control cells incubated in the presence of serum-containing medium alone for the same period of time. The presence of delipidized serum in the reaction mixture was essential; only 10% of the total counts were cell-associated when delipidized serum was omitted from the reaction mixture.

C. Discussion

The results reported in this Example suggested that HPBM, isolated into two populations based on their size and density, have equal potential to differentiate into mature macrophages. The in vitro maturation of HPBM to macrophages was associated with enhanced binding and uptake of retinol, presumably as a result of the acquisition of cell surface receptors for serum retinol-binding protein. Exposure of HPBM to rIFN-g for 72 hr led to enhanced binding of [^{3}H]ROH that was comparable to the binding activity of control HPBM cultured in vitro for 9 days. HPBM maturation induced by in vitro culture or by exposure to rIFN-g was accompanied by similar morphologic and enzymatic changes. The requirement of cell surface receptor for serum retinol-binding protein could be circumvented by direct intracellular delivery of ROH.

Recently, several reports have suggested an association between monocytic cell differentiation and induction of tissue TGase [10,17,19,21–24,34]. Freshly isolated HPBM that have very low levels of tissue TGase accumulate large amounts of this enzyme after their in vitro maturation [19,22]. Just as the two subpopulations of HPBM showed no significant difference in their ability to induce and accumu-

late tissue TGase activity during in vitro differentiation to macrophages, both fractions were equally responsive to the effect of rIFN-g in terms of augmented enzyme expression (FIG. 8). Functional heterogeneity among HPBM subpopulations isolated by similar criteria has been reported earlier. Thus, the subsets of HPBM isolated into small and large populations have been reported to produce different amounts of reactive oxygen species [37], prostaglandins [1,30], antibody dependent cell-medicated cytotoxicity [27], and tumor-cell killing [26]. This functional heterogeneity among HPBM subpopulations has been attributed to either maturational or clonal differences. The data presented herein, however, suggest no heterogeneity among HPBM subpopulations in induction of tissue TGase, a marker for monocytic cell differentiation, and equal potential for differentiating into mature macrophages. The ability of rIFN-g to enhance tissue TGase expression in both HPBM subpopulations suggests that this endogenous cytokine may play an important role in the maturation, differentiation, and expression of differentiated functions in monocytic cells.

The factors in serum responsible for induction and accumulation of tissue TGase in cultured HPBM and macrophages have been shown to be endogenous retinoids and serum retinol-binding protein [21]. Extraction of retinoids by delipidization or depletion of retinol-binding protein from the serum completely abolished its enzyme-inducing ability [19,21]. Serum retinol-binding protein is believed to be responsible for intravascular transport and delivery of retinol to specific target tissues [8,9,29,31]. Receptors for serum retinol-binding protein present on the surface of target cells are responsible for the specificity of the delivery process [9,31]. The binding of ROH-retinol-binding protein complex to cell surface receptors apparently facilitates the delivery of ROH into the interior of the cell [9,31]. At superphysiologic doses (greater than 10 nM) on the other hand, RA can enter the cells directly by simple diffusion without the participation of surface receptors for retinol-binding protein [21]. This suggested that freshly isolated HPBM probably lack the cell surface receptors for serum retinol-binding protein and therefore cannot internalize the endogenous or exogenous retinoids. Indeed, the addition of exogenous RA to HPBM cultures at doses (e.g. greater than 10 nM) at which the receptor-mediated delivery becomes irrelevant resulted in a marked induction of tissue TGase activity (FIG. 9). The enzyme-inducing ability of RA was augmented further by encapsulating RA within the liposomes and allowing its internalization via phagocytosis (FIG. 10).

Of particular interest was the effect of ROH, which, in its free form did not induce the expression of tissue TGase in freshly isolated HPBM. When ROH was encapsulated within liposomes, however, the requirement for a cell surface receptor for serum retinol-binding protein was obviated. Thus liposomal ROH induced a significant level of tissue TGase activity in HPBM (FIG. 11). This suggested an effective approach for targeting retinol or its inactive analogues to the monocytic cells with no or minimal toxic effects. Because HPBM lack cell surface receptors for serum retinol-binding protein makes administered ROH subject to nonspecific internalization by other cell types. The present studies suggested, furthermore, that interaction of ROH-retinol binding-protein complex with the cell surface receptor is required only for the intracellular delivery of retinol and that, unlike in the case of other hormones [3], ligand-receptor interaction may not require a second messenger for expression of the final event. The increase in TGase enzyme activity induced by free RA or liposome-encapsulated RA or ROH, was the result of the accumulation of enzyme protein rather than the activation of preexisting enzyme, as revealed by immunoblots of the cell lysates using an iodinated antibody to tissue TGase (FIGS. 10,11).

Preliminary data on tritiated ROH-binding (Table 5) further supported the concept that in vitro differentiation of HPBM to mature macrophages was associated with acquisition of cell surface receptors for retinol-binding protein and that treatment with rIFN-g augmented the expression of these receptors. Once the HPBM acquire these receptors, they could internalize the endogenous retinoids and induce the expression of tissue TGase. Indeed, retinoids have been shown specifically to trigger the gene for tissue TGase in myelocytic cells [23].

Impairment of macrophage function in retinoid-deficient animals has been well documented to lead to increased incidence of infections and decreased tumor-cell killing [5]. In cultures of guinea pig peritoneal macrophages, RA has been reported to increase the intracellular levels for the tumoricidal enzyme arginase [32]. The present findings that retinoids play an important role in the differentiation process of HPBM support the idea that retinoids are the important regulators of monocyte/macrophage functions.

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EXAMPLE 8

In vivo Administration

A. Materials and Methods

1. Liposomes and liposomal all-trans retinoic acid Liposomal all-trans retinoic acid was prepared from lyophilized powder in bottles containing 3 mg of all-trans retinoic acid and 45 mg of a mixture of two phospholipids, dimyristoyl lecithin and dimyristoyl phosphatidylglycerol in a 3:7 ratio (Avanti Polar Lipids, Birmingham, Ala.). Immediately before use, liposomal all-trans retinoic acid was reconstituted by adding 3 ml of normal saline to each bottle and agitating the suspension on a vortex mixer for 2–3 min. The reconstituted preparation consisted of multilamellar liposomes (average size, 3.1 μm).

2. Animals

Six-week-old Lewis rats (Charles River. Wilmington, Mass.) were used for these studies. Groups of eight female rats each were administered 5 mg/kg body weight of either all-trans retinoic acid (mixed with mineral oil) orally or liposomal all-trans retinoic acid, intravenously (in tail vein). Each rat received a total of 15 doses, twice a week, 3–4 days apart, for 7 weeks. Blood samples of 150 μl were collected from the tail vein 5, 30, 60, and 90 minutes following administration of the last dose and analyzed for all-trans retinoic acid levels by HPLC. Blood samples were also collected 60 min after administration of the first and sixth dose and analyzed for all-trans retinoic acid. Ninety minutes after the last dose, all the animals were killed and blood samples of 3 ml were collected to study the hematologic and blood chemistry parameters. Sections of tissues were collected on dry ice for further processing or were fixed in formalin for histopathologic analysis.

3. Cellular retinoic acid-binding protein (CRABP)

Liver samples were collected 90 minutes after the last dose and processed individually. Total CRABP (CRABP I and II) levels were quantitated by slab gel electrophoresis. Briefly, cytoplasmic proteins were extracted and 100–200 μg protein were incubated overnight at 4° C. in a 100 μl solution of 50 nM ^{3}[H]-all-trans retinoic acid (specific activity 49.3 Ci/mmol; and 2 mM dithiothreitol with or without 200-fold excess of unlabeled all-trans retinoic acid. Reactants were fractionated over vertical slab gel polyacrylamide electrophoresis under native conditions. After electrophoresis the gel was divided into lanes and cut into 5 mm bands; radioactivity was assessed in a liquid scintillation counter. Specific binding was determined from the radioactivity recovered with or without the 200-fold excess of unlabeled retinoid.

4. In vitro metabolism of all-trans retinoic acid

Liver samples obtained from the animals at the time of death were rinsed in ice-cold saline and homogenized individually in a 3-fold volume of 0.25M sucrose 0.05M Tris-HCl (pH 7.4) using a Teflon® glass homogenizer. Microsomes were isolated by differential centrifugation at (10,000 g for 20 min; 100,000 g, 60 min). The microsomal pellet was suspended in 0.05M Tris-HCl (pH 7.4), portioned into aliquots and stored at −70° C. Protein content was determined by Biorad Protein Assay using bovine serum albumin as the standard.

The assay buffer and conditions used for determining the ability of microsomes to metabolize [carboxyl-^{14}C] all-trans retinoic acid (specific activity 13.7 Ci/nmol) were essentially the same as those described by Van Wauwe et al., *J. Pharmac. exp. Ther.*, Vol. 245, 718. After 30 min, the reaction was stopped by cooling and the samples were lyophilized to dryness. Dried residues were extracted with methanol containing butylated hydroxyanisole (0.05%, v/v), and the extracts were evaporated and redissolved in small volumes of methanol (25–50 μl). All-trans retinoic acid and metabolites were then separated by thin layer chromatography by spotting 20,000–25,000 cpm on 0.25-mm silica-coated plastic sheets, and developing in a solution of benzene, chloroform, and water (4:1:1). The radioactive spots were located by spraying, the plates with EN^{3}Hance (New England Nuclear) and autoradiography. The radioactive bands were scraped out, extracted with Solvable (New England Nuclear) and counted in a scintillation counter. The extent of all-trans retinoic acid metabolism was determined from the proportions of cpm in appropriate zones and expressed as a percentage of the total amount of radioactivity recovered.

5. HPLC analysis

The extent of all-trans retinoic acid metabolism by isolated liver microsomes was also determined by HPLC analysis. The reactants were lyophilized and the residues were extracted twice with 2 ml of methanol containing 0.05% butylated hydroxyanisole (Sigma Chemical Co., St. Louis, Mo.). After centrifugation, the supernatants were aspirated and evaporated. The resulting pellets were re-extracted in a methanol:dichloromethane solution (75:25) and again evaporated in vacuo. More than 80% of the added all-trans retinoic acid was recovered. The final pellet was mixed with 200 μl of mobile phase for reverse-phase HPLC. A portion of each sample (150 μl) was analyzed on a 10-μm C_{18} Bondapack column (3–9×300 mm; Waters Associates, Farmingham, Mass.). Samples were eluted with a solution of methanol, water, and formic acid (60:40:0.05) containing 10 mM ammonium acetate at a flow rate of 2 ml/min. After 20 min, the solvent was changed to 100% methanol in order to elute all-trans retinoic acid.

Reverse phase HPLC was also used for determining the blood concentrations of all-trans retinoic acid. All procedures were performed in a room with the lights dimmed. Whole blood samples (200 μl) were extracted twice (1 ml each) with methanol. After centrifugation, the supernatants were vacuum-dried and the dried pellets were reconstituted in 200 μl of methanol. The recovery of all-trans retinoic acid under these conditions was calculated to be 85%±7%. The HPLC system included two pumps and a Zorbax-C8 reverse phase column (4 mm×8 cm; Supelco, Pa.). The mobile phase consisted of a linear gradient between solvent A (THF and water (25:75) containing 0.04% ammonium acetate. pH 4) and solvent B (100% THF) during a 16 minute run at a flow rate of 1.8 ml/min. The absorbance was monitored at 346 nm. Retention time for all-trans retinoic acid under these conditions was approximately 9.8 minutes.

6. Determination of P450 levels

Cytochrome P450 levels in liver microsomes were determined spectrophotometrically. The assay system is based on the carbon monoxide (CO) difference spectra of dithionite-reduced samples, assuming a value of 91 mM/cm for the molar extinction increment between 450 and 490 mμ. The P450 activity was calculated by the following formula: (change in absorbance between dithionite-reduced sample and CO sample alone)/91×1000: it was expressed as nm/mg protein.

7. Statistical analysis

The mean values for the groups were analyzed by using Student's t-test for paired samples.

B. Results

Hematologic and blood chemistry analysis of samples drawn 90 min. after administration of the last dose of liposomal or non-liposomal all-trans retinoic acid, summarized in Table 6, revealed no significant changes, except that the number of circulating segmented neutrophils was significantly decreased in animals treated with either drug formulation. This decrease in circulating neutrophils was more pronounced in rats treated with non-liposomal all-trans retinoic acid than in those treated with liposomal all-trans retinoic acid or in untreated controls ($p<0.05$). Similarly, no appreciable change was observed in most of the blood chemistry parameters studied, except both the non-liposomal all-trans retinoic acid and liposomal all-trans retinoic acid-treated rats showed slight increases in alkaline phosphatase levels (Table 6).

TABLE 6

HEMATOLOGICAL AND BLOOD CHEMISTRY PARAMETERS OF RATS FOLLOWING LONG-TERM ADMINISTRATION OF FREE ATRA AND L-ATRA*

	Control	ATRA (p.o.) (5 mg/kg body weight)	L-ATRA (i.v.)
Hematological parameters			
WBCs (× 10^3/mm^3)	5.1 ± 1.9	4.3 ± 1.3	6.3 ± 1.8
RBCs (× 10^6/mm^3)	7.1 ± 0.1	7.0 ± 0.3	6.6 ± 0.5
Hgb (gm/dl)	13.5 ± 0.4	13.3 ± 0.6	12.6 ± 1.0
Plts (× 10^3/mm^3)	607.0 ± 106	577.0 ± 252	644.0 ± 107
Segs (× 10^3/mm^3)	59.3 ± 4.9	28.6 ± 8.6	46.0 ± 14.3
Lymph (× 10^3/mm^3)	40.0 ± 4.3	69.0 ± 10.0	54.1 ± 9.0
Blood chemistry parameters			
Electrolytes (mEq/L)			
K$^+$	3.8 ± 0.2	4.1 ± 0.2	3.8 ± 0.4

TABLE 6-continued

HEMATOLOGICAL AND BLOOD CHEMISTRY PARAMETERS OF RATS FOLLOWING LONG-TERM ADMINISTRATION OF FREE ATRA AND L-ATRA*			
	Control	ATRA (p.o.) (5 mg/kg body weight)	L-ATRA (i.v.)
Na^+	142.6 ± 0.6	142.7 ± 0.9	142.5 ± 1.3
Cl^-	99.3 ± 1.5	99.0 ± 2.5	98.7 ± 3.5
Creatinine (mg %)	0.43 ± 0.05	0.56 ± 0.1	0.51 ± 0.1
BUN (mg/dl)	21.7 ± 3.2	22.7 ± 3.1	22.4 ± 4.7
SGOT (IU)	171.0 ± 33.1	144.4 ± 55.2	131.9 ± 72.1
SGPT (IU)	62.3 ± 7.6	76.5 ± 34.0	73.9 ± 17.9
Alk. Phos. (IU)	144.7 ± 5.0	188.6 ± 23.3	203.0 ± 20.7
Bilirubin (mg %)	0.2 ± 00	0.16 ± 0.05	0.13 ± 0.05

*Groups of rats were administered p.o. free ATRA or i.v L-ATRA twice a week for 7 weeks (15 doses). After the last dose, blood was collected and analyzed for various parameters. The results shown are the average values from four to eight rats ± standard deviation from the mean.
WBCs, white blood cells; RBCs, red blood cells; Hgb, hemoglobin; Plts, platelets; Segs, segmented neutrophils; Lymph, lymphocytes; BUN, blood urea nitrogen; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; Alk. Phos., alkaline phosphatase.

Microscopic examination of tissue sections from the liver, lung, spleen, brain, ovary, skin, kidney, and bone marrow of the treated rats revealed no significant changes in the histopathologic characteristics. Interestingly, spleens from seven of the eight liposomal all-trans retinoic acid-treated subjects showed the presence of numerous small vacuoles throughout the red pulp area. These structures might represent entrapped liposomes that were removed during processing. They were seen throughout the sinusoids and in phagocytes. No such vacuolization was observed in animals that were treated with non-liposomal all-trans retinoic acid or in control animals treated with saline alone.

FIG. 12(A) shows the levels of all-trans retinoic acid in the blood 60 min after oral administration of non-liposomal all-trans retinoic acid or i.v. administration of liposomal all-trans retinoic acid. In general, these blood levels were higher in rats treated with liposomal all-trans retinoic acid than in those treated with non-liposomal all-trans retinoic acid. This difference became most striking after 7 weeks of continuous drug treatment. The mean level of all-trans retinoic acid in the blood of rats treated with non-liposomal all-trans retinoic acid decreased from 3.01±0.33 μg/ml on day 1 to 1.97±0.17 μg/ml ($p<0.01$) after 7 weeks of treatment, whereas the mean blood all-trans retinoic acid levels of rats treated with liposomal all-trans retinoic acid did not change significantly. The mean all-trans retinoic acid concentration on day 1 (4.42±1.2 μg/ml) was similar to that at the end of treatment (4.41±0.2 μg/ml). Also studied was blood clearance of all-trans retinoic acid following administration of the last dose of all-trans retinoic acid. Results shown in FIG. 12(B) demonstrate that all-trans retinoic acid could be detected in the blood by HPLC 30 minutes after ingestion of non-liposomal all-trans retinoic acid. The drug reached its maximum level (2.01±0.24 μg/ml) after 60 min and remained constant for at least 90 min (1.97±0.17 μg/ml). In contrast, significantly higher concentrations of all-trans retinoic acid (7.57±1.2 ±μg/ml) were observed in the blood 5 min following i.v. administration of liposomal all-trans retinoic acid. The clearance of liposomal all-trans retinoic acid from blood occurred in two phases, the initial rapid phase ($t_{1/2}\alpha$=16 min) followed by a slower terminal phase ($t_{1/2}\beta$=55 min). Nonetheless, blood levels of the drug were significantly higher in subjects treated with liposomal all-trans retinoic acid at each time point studied ($p<0.001$) than in animals administered non-liposomal all-trans retinoic acid.

Particularly addressing FIG. 12: Blood concentrations of all-trans retinoic acid in rats after 7 weeks treatment with non-liposomal all-trans retinoic acid or liposomal all-trans retinoic acid are presented. FIG. 12(A) presents data from groups of eight rats administered (5 mg/kg body weight) either p.o. non-liposomal all-trans retinoic acid (cross-hatched bars) or i.v. liposomal all-trans retinoic acid (solid bars) twice a week for a total of 7 weeks. Blood samples (200 μl) were collected 60 min after the administration of the first, sixth, and fifteenth doses, and 150 μl aliquots of the blood were analyzed for all-trans retinoic acid by HPLC. In FIG. 12(B) data is presented following administration of the last dose. Blood samples were collected from animals treated with non-liposomal all-trans retinoic acid (open circles) or liposomal all-trans retinoic acid (solid dots) at indicated time intervals and analyzed by HPLC for all-trans retinoic acid content. The results shown are mean plasma drug concentrations in six rats±S.D.

Because cytochrome P450-dependent accelerated catabolism and induction of CRABP have been implicated in the acquisition of clinical resistance to all-trans retinoic acid, a determination was made that the CRABP and cytochrome P450 levels in liver tissues of rats that had been treated with either all-trans retinoic acid formulation was mad. No appreciable differences in CRABP levels were observed between liver samples of rats that had been treated with non-liposomal all-trans retinoic acid or liposomal all-trans retinoic acid and untreated controls. Similarly, there were no significant changes in total cytochrome P450 levels in liver microsomes from rats treated with non-liposomal all-trans retinoic acid (0.63±0.13 nmol/mg; n=7) or liposomal all-trans retinoic acid (0.59±0.01 nmol/mg; n=7) or untreated rats (0.68±0.15 nmol/mg; n=6).

In vitro, however, the liver microsomes isolated from rats that were treated with non-liposomal all-trans retinoic acid exhibited significant rapid catabolism of all-trans retinoic acid. Incubation of [^{14}C]all-trans retinoic acid with isolated liver microsomes in the presence of NADPH resulted in rapid conversion of all-trans retinoic acid into two polar products as determined by thin layer chromatography. Incubation under similar conditions of liver microsomes from untreated rats or rats treated with liposomal all-trans retinoic acid revealed a significantly slower rate of metabolism of all-trans retinoic acid into these polar metabolites. When combined, these metabolites accounted for about 33±0.8% of the microsomes from untreated rats and 28.8±2.57% of those from liposomal all-trans retinoic acid-treated rats, whereas they accounted for 57±11.2% of the microsomes from animals treated with non-liposomal all-trans retinoic acid. (FIG. 13(A)) Individual values for intact all-trans retinoic acid and its polar metabolites generated in the presence of NADPH by liver microsomes that were isolated from five different rats treated with either non-liposomal all-trans retinoic acid or liposomal all-trans retinoic acid or from three untreated rats are shown in FIG. 13 (B). Microsomes from all liposomal all-trans retinoic acid-treated and control animals induced much slower catabolism of all-trans retinoic acid than those from rats administered non-liposomal all-trans retinoic acid (FIG. 13(B)). Liver microsomes isolated from rats that were treated with "empty liposomes" without all-trans retinoic acid showed rates of conversion of all-trans retinoic acid to its metabolites similar to those of the untreated controls. The reaction products generated by incubating all-trans retinoic acid in the presence of NADPH and liver microsomes were further analyzed by reverse phase HPLC. Results of that HPLC analysis demonstrated that microsomes from rats treated with non-

liposomal all-trans retinoic acid converted the drug into four major products (retention times, 7.5–11.5 min). Two of the metabolites were eluted at the same positions as authentic 4-keto all-trans retinoic acid (retention time, 7.8 min) and 4-hydroxy all-trans retinoic acid (retention time, 9.5 min). Incubation of microsomes from rats injected with liposomal all-trans retinoic acid also converted all-trans retinoic acid into polar metabolites, but these metabolites were different, quantitatively and to some extent qualitatively, from those in the group treated with non-liposomal all-trans retinoic acid. For example, the metabolite that eluted at 11.1 min from the non-liposomal all-trans retinoic acid microsome reaction mixture was not seen in the liposomal all-trans retinoic acid microsome reaction mixture. Similarly, the amounts of three other products (retention times, 7.8–9.6 min), were much smaller in the reaction mixture incubated with microsomes from liposomal all-trans retinoic acid-treated rats.

Addressing FIG. 13: The effect of long-term all-trans retinoic acid administration on drug metabolism by liver microsomes is presented. FIG. 13(A), at the end of the 7 week treatment period, animals were killed and their liver microsomes isolated. The ability of microsomes to metabolize in vitro [^{14}C]all-trans retinoic acid was then determined by incubating microsomes in the presence of NADPH and radiolabeled all-trans retinoic acid (50 nM). The reaction products were fractionated by thin layer chromatography and extent of drug metabolism was determined by counting the metabolite fractions. Results are expressed as a percentage of all-trans retinoic acid metabolized to polar products (cross-hatched bars) or percentage of all-trans retinoic acid remaining intact (solid bars). The vales shown are averages from five rats±S.D. FIG. 13(B) presents radioactivity (cpm) recovered from intact all-trans retinoic acid (lane 1) or its polar metabolites (lane 2), as discussed in FIG. 13(A), were plotted individually for five different rats.

Non-liposomal all-trans retinoic acid has been ineffective in permanently maintaining the remission state of acute promyelocytic leukemia ("APL"). Even when all-trans retinoic acid administration is continued after remission has been achieved, many APL patients still experience relapse. Clearly, some mechanism of resistance develops in relapsed patients whereby the ability of all-trans retinoic acid to induce cellular differentiation is diminished substantially. Several in vitro studies have attempted to explain the evolution of this resistance mechanism, which can be induced in culture after continuous exposure to elevated concentrations of retinoid or carotenoid. Interesting recent clinical pharmacological evidence regarding all-trans retinoic acid resistance (Muindi et al., *Blood*, 79:299 (1992); *Cancer Res*., 52:2138 (1992)) concluded that the reason for the eventual occurrence of this retinoid resistance during all-trans retinoic acid therapy is progressively decreasing plasma drug concentration levels. In most subjects the onset of the decrease in plasma drug concentration levels is within 2–6 weeks after beginning treatment. Although these lower all-trans retinoic acid plasma levels cannot sustain the differentiation effects on leukemic cells in vivo, in culture the leukemic cells from these patients continue to demonstrate cytodifferentiation sensitivity to all-trans retinoic acid. This resistance is not seen with other retinoids such as isotretinoin or etretinate.

An advantage of the liposomal all-trans retinoic acid of this invention is that the lipid formulation bypasses the clearance mechanism that evolves in the livers of patients treated with the oral formulation. Liposomal formulation is thus not be subject to the same relapse rates as have been demonstrated in clinical trials of the non-liposomal formulation. In addition, the toxic effects of liposomal all-trans retinoic acid should be less severe than those associated with non-liposomal all-trans retinoic acid because liposome encapsulation of all-trans retinoic acid decreases direct exposure of the drug during circulation to levels below the orally administered toxic dose. The latter allows greater total exposure of the drug on initial dose accompanied by slower clearance of the all-trans retinoic acid from the site of stem cell seeding.

All-trans retinoic acid is metabolized by a hydroxylation reaction of the cyclohexenyl ring, to produce 4-hydroxy metabolites which are further oxidized to the 4-oxo metabolites. The hydroxylation of all-trans retinoic acid to the 4-oxo-all-trans retinoic acid metabolite is mediated by cytochrome P450-dependent enzymes. The most favored explanation of the pharmacological mechanism of all-trans retinoic acid resistance is that continuous all-trans retinoic acid treatment acts to induce catabolic enzymes that are responsible for conversion of the drug. Animal studies in which all-trans retinoic acid was administered in combination with cytochrome P450 enzyme inhibitors (e.g., ketoconazole or liarozole) showed a significant prolonging of the all-trans retinoic acid plasma half life, thereby supporting the contention of accelerated enzymatic degradation. The results reported here confirm the previous observations that chronic oral administration of all-trans retinoic acid in rats results in decreased drug plasma concentrations, whereas i.v. administration of liposomal all-trans retinoic acid at a similar dose and regimen did not alter the pharmacological behavior of the drug and the blood levels remained stable throughout the study period (FIG. 12). The observed differences in pharmacological behavior of the two formulations were consistent with induction of an enzymatic process. Although no differences were observed in total P450 levels in rats treated with either formulation, microsomes from rats treated with non-liposomal all-trans retinoic acid metabolized the drug much more rapidly than those from rats treated with liposomal all-trans retinoic acid (FIG. 13).

Another factor that might contribute to the retinoid relapse phenomenon involves the role of high affinity retinoic acid-binding, proteins CRABP I and II. These proteins are believed to mediate the transfer of the retinoid from cytoplasm to the nucleus of the cell. Increased levels of CRABP may cause the pooling of retinoids in tissues resulting in low plasma levels and accelerated clearance of the drug from the circulation. In normal body tissues the expression of CRABP is thought to increase with continuous exposure to retinoids. An increase in CRABP has been documented in human skin as a result of repeated topical application of all-trans retinoic acid. A similar increase in skin CRABP levels was also observed by Adamson et al. in rhesus monkeys following chronic i.v. administration of all-trans retinoic acid.

These authors concluded that the increase in CRABP expression was not related to the increase in plasma drug clearance observed with continuous all-trans retinoic acid administration, but rather was related to catabolic enzyme induction. In the present data, no increase in levels of liver CRABP was observed in rats administered either non-liposomal all-trans retinoic acid or liposomal all-trans retinoic acid on a continuous basis.

The results of Example 8 study, coupled with the following data obtained in clinical trials discloses that long term oral administration of all-trans retinoic acid is associated with the rapid clearance of the drug from plasma that, in turn, contributes to the relapse of the disease in APL patients, strongly supports the rationale of using liposomal all-trans retinoic acid to induce long-term remissions in APL patients.

EXAMPLE 9

In vivo i.v. liposomal all-trans retinoic acid Subjects with hematological malignancies

Liposomal all-trans retinoic acid was administered i.v. over one-half hour every other day for 28 days to human subjects with hematological malignancies, including T cell cutaneous lymphoma and APL. Doses investigated were 15 mg/M^2 (5 points), 30 mg/M^2 (3 points), 60 mg/m^2 (3 points), 75 mg/M^2 (7 points), and 90 mg/M^2 (3 points). No dose limiting toxicity has been observed.

Two efficacious responses have been observed. One modestly favorable response was in a subject with T cell cutaneous lymphoma in a patient considered resistant to oral all-trans retinoic acid. This patient is presently receiving a second 28 day treatment cycle.

A subject with APL in first relapse 10 months after receiving oral all-trans retinoic acid in three weeks of the liposomal treatment of the present invention displayed a rising white count and evidence of increased cellular differentiation in both the blood and the marrow.

Pharmacokinetic drug level data was also compared to published data for all-trans retinoic acid. As taken form Trump et al., *ASCO Droc.*, Vol. 13, page 241 (1994) referencing a with prostate cancer, all-trans retinoic acid (non-liposomal) administered orally at 50 mg/M^2, twice per day for 14 days yielded a C_{max} in ng/ml on day 1 of 307 and day 14 of 144. The AUC in μg hr/ml was 0.693 on day 1 and 0.250 on day 14. A substantially distinct result was obtained using the liposomal all-trans retinoic acid of the present invention in one patient administered 60 mg/M^2 every other day for 15 doses i.v. The C_{b0} (the concentration in blood at the conclusion of i.v. administration, time 0) in μg/ml was 6.8 on day one and 7.0 on day 15 after the eighth dose. The AUC in μg/ml×min was 466 on day 1 and 580 on day 15. Converting to μg hr/ml these values are 7.76 and 9.66 respectively.

The clearance of liposomal all-trans retinoic acid was found to closely fit (r^2>0.9) a two compartment mathematical model in 14 of 22 complete analyses, and was best fit by a one compartment model in 8 of 22 studies. Where present, alpha-phase half-lives ranged from 56±20 minutes at the 15 mg/m^2 dose level to 116±43 minutes at the highest level analyzed, 75 mg/M^2. There were no statistically significant differences (Student's t-test at ρ<0.05) in the calculated half lives between day 1 and 15. In addition, there were no significant differences in half-lives at the different dose levels studied.

The apparent volume of distribution (V_d) was 25±2 liters at the 15 mg/m^2 dose level (day 1) suggesting rapid distribution into a space approximating total body water. As observed with the half-life data, there were no statistically significant differences in V_d between subjects at the different dose levels or between patients treated on day 1 or 15. Both the C_{b0} and the extrapolated area under the concentration curve (C_{xt}) were found to increase proportionately over the dose range studied. Further, this range was not statistically different from Day 1 to 15. These pharmacokinetic studies disclose that the liposomal formulation of the present invention maintains blood level and does not exhibit the reduction in blood levels(retinoid resistance), or other parameters associated with prolonged oral administration of all-trans retinoic acid. Further, in the instant study, the absence of dose-dependent and time-dependent increases in pharmacokinetic parameters indicate no apparent saturation of drug clearance mechanisms.

The preceding description is intended to illustrate specific embodiments of the present invention. It is not intended to be an exhaustive list of all possible embodiments. Person skilled in the relevant field will recognize that modifications could be made to the specific embodiments which have been disclosed, that would remain within the scope of the invention.

We claim:

1. A method of inhibiting the growth of retinoic acid responsive cancer cells and avoiding all-trans retinoic acid resistance, said method, comprising administering to a living subject a therapeutically effective amount of a retinoic acid composition which comprises all-trans retinoic acid, liposomes whose lipid component consists essentially of dimyristoyl phosphatidyl choline, and a triglyceride; where the retinoic acid is substantially uniformly distributed with the dimyristoyl phosphatidyl choline in the liposomes, where the molar ratio of retinoic acid to dimyristoyl phosphatidyl choline is at least about 15:85, where the triglyceride is at least about 15% by weight of the composition, and where the composition is stable in an aqueous environment.

2. The composition of claim 1 wherein the triglyceride from is soy bean oil.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,811,119

DATED : September 22, 1998

INVENTOR(S) : Mehta et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In claim 2, column 28, line 43, change "composition" to --method--.

Signed and Sealed this

Twenty-second Day of December, 1998

Attest:

Bruce Lehman

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

C19

CANCER TREATMENT REVIEWS 1999; 25: 229–235
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LABORATORY – CLINIC INTERFACE

CANCER TREATMENT REVIEWS

Fenretinide and its relation to cancer

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Retinoids, natural or synthetic substances which have vitamin A activity, have a well-known reputation for their antitumour and differention-inducing activity in vitro and in vivo. More than 1500 retinoids have been tested so far but very few of them have been entered into clinical trials because of their side-effects. All-trans-N-(4-hydroxyphenyl)retinamide (4HPR or fenretinide) is a synthetic retinoid that is reported to have fewer side-effects compared to naturally occurring retinoids such as all-trans retinoic acid (ATRA) and 9-cis retinoic acid. In addition, fenretinide has been shown to induce cell death (apoptosis) even in ATRA-resistant cell lines. Although the mechanism by which fenretinide acts is not entirely known it is considered to be a promising drug and seems to induce apoptosis via different pathway(s) from classical retinoids. In this reveiw, we discuss possible mechanisms of fenretinide action and summarize results of clinical trials.

INTRODUCTION

It has been known for many years that vitamin A is essential for normal differentiation and maintenance of epithelial tissues. In addition, vitamin A, usually known as *retinol*, and its *derivatives*, called retinoids, have an increasing importance as chemopreventive agents through their antiproliferative and/or differentiating effects on certain types of tumours (1). Recently, some retinoids, such as fenretinide, have also been shown to induce cell death by apoptosis in some cancer cell lines (see below), which can be considered as a contribution to their total antitumour effects. However, neither the mechanisms of their chemopreventive effects nor their effects on apoptosis are fully understood.

So far, more than 1500 retinoids have been produced and tested under in-vivo or in-vitro conditions. All-trans-N-(4-hydroxyphenyl)retinamide (4HPR or fenretinide), first produced in the USA in the 1960s, is one such compound (Figure 1). As can be seen from its structure, it is a synthetic amide derived from all-trans-retinoic acid (ATRA). According to the results of various in-vitro cell culture studies, fenretinide seems not only to inhibit the proliferation of cancer cells but also can cause cell death by inducing apoptosis in T lymphoma and T lymphoblastoid leukemia cells (2), human breast cancer cells (3), small cell lung cancer cell lines (4), non-small cell lung cancer cells (5), prostate adenocarcinoma cells (6), head and neck squamous cell carcinoma (SCC) cells (7,8), and melanoma cells (9).

Fenretinide has also been tested in animal models in terms of its antitumour effect *in vivo*. Fenretinide inhibited the proliferation and also induced apoptosis of colon adenoma cells in male F344 rats (10), reduced the yield of carcinogen-induced colon tumours in rats (11,12), induced complete regression of carcinogen-induced first mammary tumours in 22% of animals and partial regression in a further 19% of animals (13), inhibited the induction of mammary adenocarcinomas compared with carcinogen controls in female rats (14), suppressed bladder carcinoma induction by N-butyl-N-(4-hydoxybutyl) nitrosamine (15), exerted chemopreventive effects against exogenous and endogenous rat liver carcinogenesis (16), reduced carcinogen-induced pancreatic adenomas in female hamsters (17), inhibited tumour progression and multiplicity in a two-stage skin cancer model in CD-1 and SENCAR mice (18), reduced naturally occurring skin tumours in ACI/segHapBR rats (19), and if given via the peritoneal cavity rather than by mouth, significantly increased the survival time of mice with ovarian carcinoma (20). In contrast to these beneficial effects, fenretinide did not inhibit the total tumour formation in the lung of female A/J mice in which carcinogenesis was chemically induced by tobacco nitrosamines (21). In addition, there was no protective effect against the induction

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Volume 25 Number 4 August 1999

Contents

CANCER TREATMENT REVIEWS

Continued over

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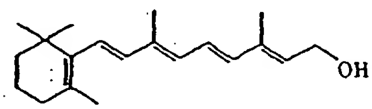
(a)



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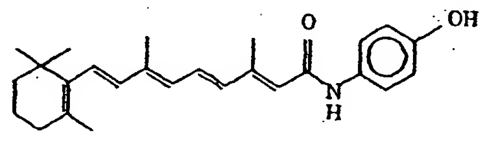

Figure 1 Structures of vitamin A (retinol) (A) and fenretinide (B)

of chemical carcinogenesis of prostate in Wistar-Unilever rats (22). In another study, although fenretinide reduced the incidence of prostate tumors in treated rats compared to controls (27.5 versus 43.2%), the difference was not statistically significant (19). However, fenretinide considerably reduced the development of prostate cancer in the Lobund-Wistar rat model to about one fourth (21 versus 88%) (23).

On the basis of these data, it was decided that fenretinide was a promising candidate for chemoprevention trials. It has therefore been entered into a number of clinical trials in oncology, mainly because of its anticancer properties coupled with lower side effects compared to other retinoids (24,25).

METABOLISM AND PHARMACOKINETICS OF FENRETINIDE

It seems that fenretinide has significantly different properties when compared with other retinoids in terms of its mode of storage and plasma half-life. The main difference is the absence of hepatic accumulation of fenretinide, implying reduced liver toxicity. Fenretinide did not cause any detectable increase in hepatic retinoid levels in rats after oral administration (26) and in the same study, fenretinide was also found to be selectively accumulated in the mammary glands, which makes it an attractive agent for chemoprevention of breast cancer. The absence of hepatic accumulation was also confirmed by another study although transient increases in liver function tests occurred after each dose (27). In this study, the terminal plasma half-life of fenretinide was found to be 12 hours which is much longer than its natural analog, ATRA. This finding may also make fenretinide more preferable. In contrast, in another study (28) performed on both rats and mice, liver was the second organ in both species accumulating the highest concentrations of fenretinide, with the bladder and mammary glands the first and third highest respectively. In a trial of fenretinide in women who had been operated on for breast cancer, a rise in liver enzymes to two to four times higher than normal levels occurred in only seven of 101 patients without clinically significant liver toxicity (29).

Fenretinide is mainly metabolized to a lipophilic compound, N-(4-methoxyphenyl)-all-trans-retinamide (MPR), which is the major circulating fenretinide metabolite, as well as polar retinamides, including HPR-O-glucuronide (30). Polar metabolites are excreted into urine and bile, while the nonpolar metabolite, MPR, accumulates in tissues including fat, prostate, skeletal muscles, liver and intestines. In another study in female mice after an oral treatment with 10 mg/kg, the highest levels of MPR were detected in liver and mammary tissue (31).

Whether or not MPR is an active metabolite seems to be controversial. It was tested as an active substance (32). Moreover, MPR was stated to be equipotent to fenretinide in reversing keratinization of retinoid-deficient hamster trachea *in vitro* (30). Conversely however, MPR was biologically inert in one study (33). In addition, it has recently been reported not to be an active metabolite of fenretinide as it failed to inhibit the growth of fenretinide-resistant UISO-Mel-6 cells, and showed no dose-dependent inhibition of fenretinide-sensitive breast carcinoma and melanoma cell lines (34). It was also suggested by the same authors that although MPR is not an active metabolite of fenretinide, detection of this metabolite in malignant cells might serve as an indirect biomarker for predicting the response of cells to fenretinide because MPR was detected only in cells sensitive to fenretinide. MPR is also a major determinant of fenretinide-induced reductions in plasma insulin-like growth factor-1 (35) and retinol (36).

Some studies have shown that fenretinide may affect retinol metabolism in the liver. For example, it seems to be able to induce the secretion of RBP, retinol binding protein, the protein which carries retinol in the plasma from the liver into the bloodstream, and leads to rapid RBP accumulation in the kidney (37) In another study (38) it was concluded that fenretinide partially blocks the secretion of the retinol-RBP complex from the liver and other tissues, and thus depresses plasma concentrations of both vitamin A and RBP. Interestingly, it was also demonstrated that fenretinide induced liver RBP secretion in vitamin A deficient rats but depressed it in those with adequate levels of vitamin A (39).

It has recently been claimed that fenretinide itself binds to RBP, and thereby induces secretion of RBP in HepG2 cells, and that the secreted fenretinide-RBP

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complex has a reduced affinity for TTR (transthyretin). This observation may explain the fenretinide-induced reduction of plasma retinol observed in in-vivo studies (40). Plasma retinol reduction after fenretinide treatment was also observed in human subjects. For example, in one of the chemopreventive trials, fenretinide treatment resulted in reduction in the levels of plasma retinol (41). However, baseline retinol plasma concentrations recovered 1 month after treatment interruption. Retinol levels were also found to have a negative relationship with fenretinide dose (42) This decrease in plasma retinol might be responsible for the improvements observed in patients with actinic keratoses who applied topical fenretinide twice daily for 3 months (43). It has also been suggested that fenretinide may have clinical utility because of its ability to increase the biological half-life of ATRA (44).

MECHANISM OF BIOLOGICAL ACTIVITY AND APOPTOSIS-INDUCING EFFECT OF FENRETINIDE

The biological activities, including the anti-growth effect, of fenretinide could be mediated by interaction with the nuclear retinoid receptors. However, it is not entirely clear whether fenretinide can in fact activate these receptors. In transactivation assays, it was observed that fenretinide was a potent transactivator with RARγ and a moderate activator with RARβ, but did not interact with RARα and RXRα. Furthermore, optimal receptor activation has been found at fenretinide concentrations which correspond with those required for its activities as a potent growth inhibitor and inducer of apoptosis (45).

Growth inhibition by fenretinide as well as ATRA was stated to be correlated with the induction of the RARβ2 gene in GLC82 (lung adenocarcinoma) and BGC823 (stomach adenocarcinoma) cells. Furthermore, exogenous RARβ2 expression potentiated fenretinide-induced growth inhibition, suggesting that fenretinide acts at least in part via the RARβ receptor (46). In contrast, in one study exposure to fenretinide resulted in the generation of DNA fragmentation with subsequent cell death in both ATRA-positive oestrogen receptor (ER)-positive as well as ATRA-refractory ER-negative breast carcinoma cell lines. Consequently, it was suggested that fenretinide might mediate its biological actions via a novel pathway(s) not involving the classical retinoid receptor pathways (47). Taken together, because of different retinoid receptor expressions in various tissues, the anti-tumour effect of fenretinide may be limited to some tissues. Its mode of action may also be different from that of ATRA. The observed differential responsiveness of a number of haemopoietic cell lines, which are resistant to ATRA, but which respond to fenretinide, also indicates that these compounds may act through different receptors (48). It has been suggested that fenretinide and indeed ATRA, at least in terms of mediating growth inhibition, might act by different mechanisms (49). It has also been demonstrated that contrary to the differentiation-promoting activity of ATRA, fenretinide dramatically suppressed neuroblastoma (NB) cell growth by inducing apoptosis (50). As mentioned above, fenretinide has been shown to induce apoptosis in head and neck SCC cells whereas ATRA did not appear to have any apoptosis-inducing effect on the same cell lines (7). Indeed, it has been suggested that ATRA can promote a semi-apoptosis-resistant status in neutrophils, possibly through the overexpression of the Bcl-2 gene, whose product is known to be an apoptosis-inhibiting protein, while fenretinide can cause apoptosis (51).

As mentioned above, the mechanism of apoptosis induction by fenretinide as well as by some other retinoids is not well understood. One possibility is that fenretinide can enhance the generation of reactive oxygen species which may then be involved in the apoptotic pathway (52). This phenomenon seems to be restricted to fenretinide and is not observed with other retinoids, including ATRA and 9-cis-retinoic acid (9-cis-RA) (52,53). Another possibility is that fenretinide may affect the cell survival signals received via integrins from the extracellular matrix. It is thought that abrogation of cell adhesion by fenretinide through down-modulation of integrin receptors plays a crucial role in the induction of neuroblastoma programmed cell death (54). However, in this case the same effect was also achieved by ATRA. Fenretinide may also affect the cell cytoskeleton. For example, it has been suggested that fenretinide might trigger apoptosis by inducing overall cytoarchitectural changes and specific DNA fragmentation subsequent to increased turnover of the protein actin in HL-60 cells (51).

In general, retinoids are well known for their differentiating effects, but the effect of fenretinide on differentiation has only been observed in a few cases. For example, fenretinide completely reversed keratinization in squamous metaplasia of hamster tracheal organ cultures resulting from vitamin A deficiency (26), while it either did not show (51) or had a poor (44) differentiating effect on the human promyelocytic leukemia cell lines, HL-60 and NB4, respectively. Furthermore, it was also demonstrated that, contrary to the differentiation-promoting activity of ATRA, fenretinide dramatically suppressed neuroblastoma cell growth by inducing programmed cell death (50). However, fenretinide up-regulated the expression of several differentiation markers

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(e.g., class 1 HLA, laminin, and β1 integrin chain), and down-regulated expression of molecules associated with tumour progression, including the p185/HER2 oncoprotein, the epidermal growth factor receptor, and the M(r) 67,000 laminin receptor in breast cancer cell lines (55).

HPR can induce the expression of transforming growth factor-β1 (TGF-β1) in association with the induction of apoptosis (56). It may also regulate the activity of certain cell surface receptors. Thus, fenretinide as well as ATRA treatment of esophageal squamous carcinoma cell lines resulted in downregulation of the epidermal growth factor receptor (EGFR) which is known to bind proliferative ligands such as epidermal growth factor and transforming growth factor α (TGF-α) (57).

The fenretinide concentration used may be the critical point at which cells have to decide whether to undergo either cell death by apoptosis and/or necrosis or cytostasis. Moreover, the type of cell, normal or transformed, also seems to be important in this respect. In one study, fenretinide failed to inhibit the growth of some cervical carcinoma cell lines when used at 1 M but when used at 5 or 10 M, it induced apoptosis (58). In contrast, we found that fenretinide at 1 M inhibited the growth of cell line A431, a squamous cell carcinoma cell line (unpublished observations). We also found that the growth of skin fibroblasts was not affected by the same concentration as was used for A431 cells.

FENRETINIDE AND APOPTOSIS-RELATED GENE PRODUCTS

There may be a relationship between fenretinide and apoptosis-related gene products, such as p53 and the Bcl-2 family. An extensive study on these gene products was carried out on human leukaemic cell lines and although changes in Bcl-2, p53, and c-myc expression were observed in cells treated with HPR, the time-course of these events suggested that fenretinide-triggered apoptosis was not directly controlled by these genes and that ectopic overexpression of Bcl-2 markedly delayed the onset of apoptosis, rather than protecting cells from death by fenretinide (59). A study on human breast cancer cells showed that fenretinide treatment resulted in decreased Bcl-2 mRNA levels but not Bax mRNA levels, and induced apoptosis in both oestrogen receptor-positive and -negative cells (3). However, it was also found that the fenretinide failed to modulate cellular levels of the Bcl-2 and Bax proteins (52). In another study, p53 and pRB were suppressed in response to fenretinide in the androgen-independent human prostatic JCA-1 cells (60).

SYNERGISTIC COMBINATIONS OF FENRETINIDE WITH ANTI-CANCER DRUGS

Fenretinide has been used in a number of synergistic combinations in order to try to increase its antitumor efficacy on different types of cancer cells. For example, it seemed that fenretinide potentiated the effects of cisplatin in ovarian carcinoma (20,61). It has also been combined with glucarate and this combination therapy inhibited the growth of human mammary tumour cells grown in athymic mice, the growth of rat mammary tumours in germfree rats, and the hormone-independent MTW 9a/R rat mammary tumour (62). Combinations of fenretinide with either tamoxifen or various cytokines also seems promising.

One study (63) concluded that fenretinide induces the expression of TGF β-1 in association with the induction of apoptosis in prostate cancer cells in vitro, and furthermore, fenretinide-induced cytotoxicity was abrogated by the addition of anti- TGF β-1 antibody. It was also reported that the combination of fenretinide (0.1 μM) and tamoxifen (1 μM) or IFN- γ (500 IU/ml) generally had additive or synergistic effects on all the breast cancer cell lines investigated (64). In an in-vivo study (65), a combination of fenretinide with tamoxifen was found to be effective in inhibiting MNU-induced adenocarcinomas in female Sprague-Dawley rats, and the reduction in tumour incidence was greater for this combination than for either agent alone. Combinations of fenretinide and tamoxifen can be safely administered to women (66). This combination was also found to be more effective than treatment with either of the agents alone in inhibiting of growth of human colon cancer cells in vitro (67). Fenretinide may have a synergistic effect with irradiation too. Low dose fenretinide combined with low dose gamma-irradiation seemed to have a synergistic effect on apoptosis, with the number of apoptotic cells increased by more than 30% (68).

TOXICITY AND CLINICAL TRIALS WITH FENRETINIDE

In Phase I/II trials, fenretinide seemed to be well-tolerated with only minimal or mild toxicity depending on the dose used. There are currently a number of National Cancer Institute (NCI)-sponsored clinical trials still in progress (69). It was previously reported that the recommended dose for chemoprevention trials of HPR orally was 200 mg per day (70).

A Phase I/II trial in women with oestrogen receptor (ER)-positive or progesterone receptor (PR)-positive,

previously untreated metastatic breast cancer was performed to evaluate the combined toxicity of tamoxifen plus fenretinide (71). There were no significant adverse effects on renal, hepatic, hematologic, or lipid values. Nyctalopia, photophobia, cheilitis, and pruritus (typical features of retinoid treatment) were not observed. Improvement or stabilization of disease occurred in 12 of 15 patients. Therefore it was concluded by authors that tamoxifen administered with fenretinide is nontoxic. Phase III trials of tamoxifen versus tamoxifen plus fenretinide are now warranted. Toxicity was mild in another phase II study of fenretinide involving 31 patients with either advanced breast cancer or melanoma (72).

Toxicity and the feasibility of using fenretinide as a chemoprevention agent in men at high risk for prostate cancer has been evaluated in 22 patients. Fenretinide was well-tolerated, and no major toxicity was associated with its use (73). However, because eight patients with negative prestudy biopsies had positive prostate biopsies prior to, or at the time of, their 12th cycle evaluation, the study was closed early.

In one chemopreventive trial, fenretinide was shown to prevent recurrences and the development of new sites of oral leukoplakia. Again the drug was well tolerated (74,75). However, fenretinide was not effective in a Phase II trial in the treatment of myelodysplasia and it was even stated that in some patients fenretinide may enhance leukaemic progression (76). This result may not be all that surprising because fenretinide tends to accumulate especially in solid tissues such as breast, prostate, and, to some extent but not at a toxic level, in the liver.

Another chemopreventive trial aiming at preventing contralateral primaries in women already treated for breast cancer, whose risk is 0.8% per year within 10 years from primary treatment, started in 1987: accrual of patients was closed in 1993 with a total of 2972 patients, and the study is still ongoing (24). The other trial is the basal cell carcinoma study, which started in January 1990. The study is ongoing (77).

CONCLUSION

It may be concluded from both in-vivo and in-vitro studies that fenretinide is well-tolerated and has some anti-cancer properties, including anti-proliferative and apoptosis-including effects. More clinical trials are obviously warranted but much work remains to be carried out in order to elucidate its mechanism(s) of action.

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United States Patent [19]

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[54] METHOD AND COMPOSITION FOR ACHIEVING CHEMOTHERAPEUTIC ACTIVITY

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Walaszek et al., IRCS Medical Science, 1986; 14:677-678.

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Moon et al., Cancer Res., 1979; 39:1339-1349.

Rustin et al., Brit. J. Cancer, 1985; 51:443-445.

Welsch et al., *Diet, Nutrition, and Cancer*, 1986; (B. S. Reddy and L. A. Cohen eds.) CRS Press, Boca Raton, FL pp. 1-21 (1986).

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Moon et al., Cancer Res., 1976; 36:2626.

Abou-Issa et al., Proc. Natl. ACad. Sci., (U.S.) 1988; 85;4181-4184.

Primary Examiner—Mary C. Lee

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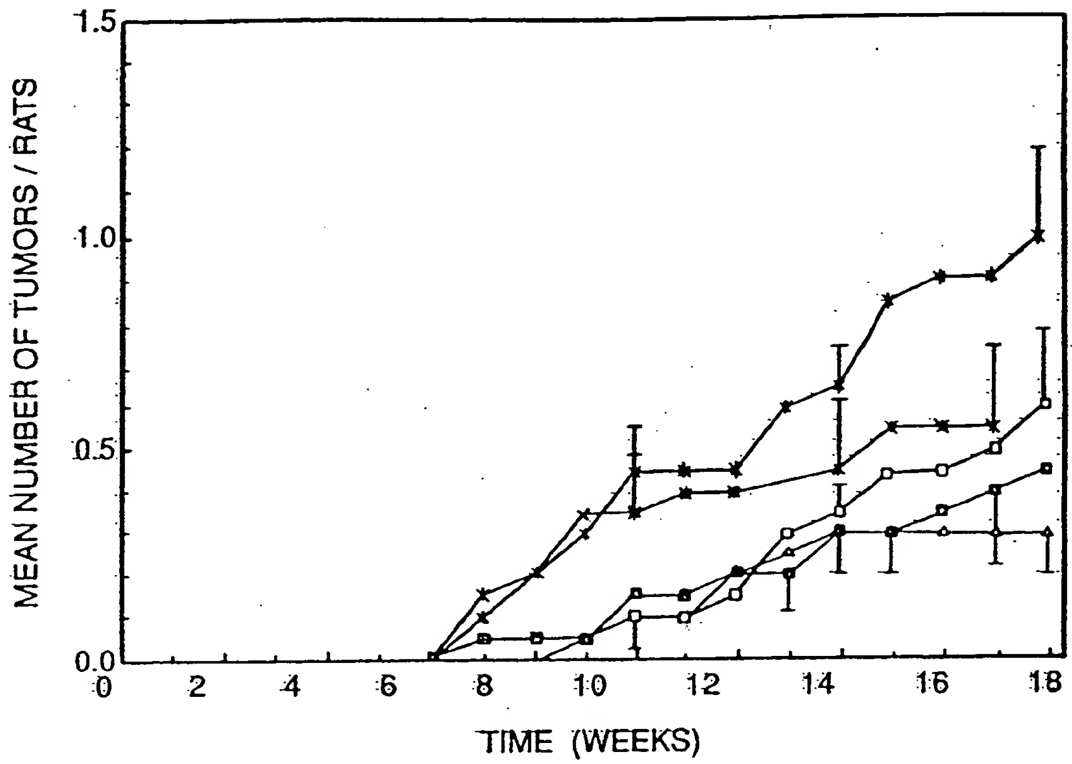
[57] **ABSTRACT**

The present invention describes a dietary supplement for achieving a very high degree of chemotherapeutic activity through the synergistic combination of a low suboptimal dose of a D-glucarolactone-based dietary anticarcinogen (calcium glucarate) with a low suboptimal dose of retinoid-based anticarcinogen (4-hydroxyphenyl retinamide).

7 Claims, 5 Drawing Sheets



EFFECT OF DIETS ON MEAN NUMBER OF TUMORS / RAT

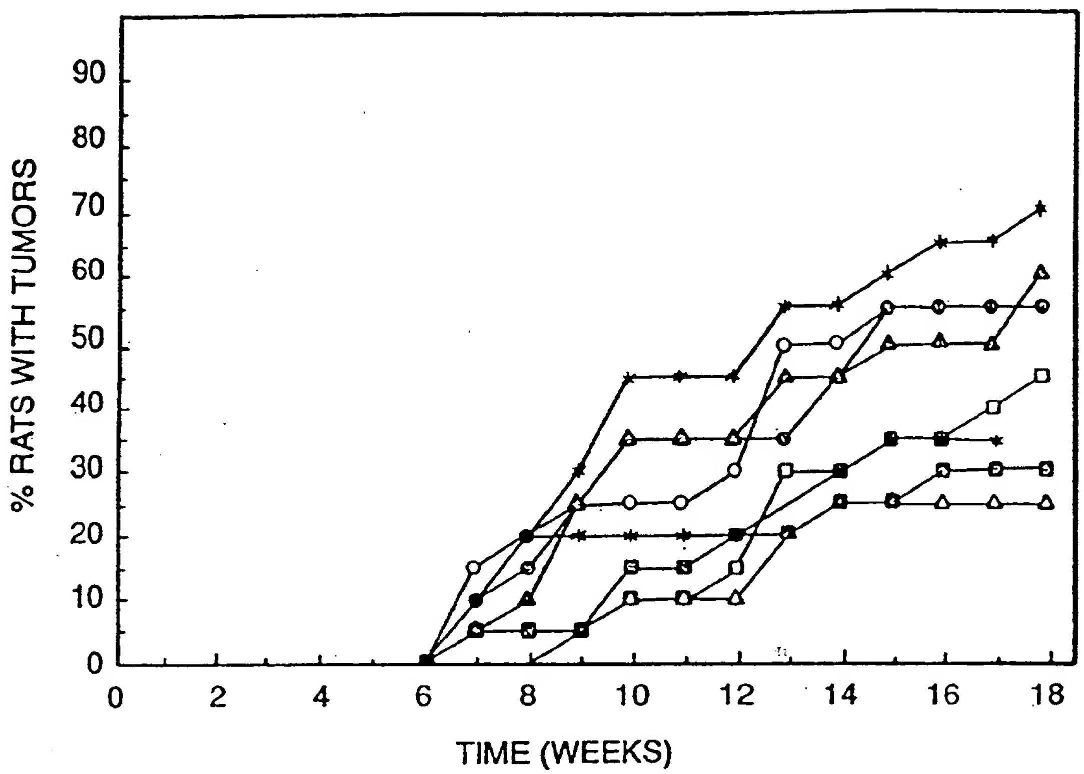
✱ CONTROLS (CHOWDIET)

△ 1.5 % HPR

□ 0.75 mmol/kg HPR + 1 % CGT

▩ 0.75 mmol/kg HPR + 2 % CGT

FIG. 1



EFFECT OF DIETS ON % RATS WITH TUMORS

✳ CONTROLS (CHOWDIET)

○ 1 % CGT

◍ 2 % CGT

□ 0.75 mmol/kg HPR + 1 % CGT

▩ 0.75 mmol/kg HPR + 2 % CGT

△ 1.5 mmol/kg HPR

▲ 0.75 mmol/kg HPR

FIG. 2

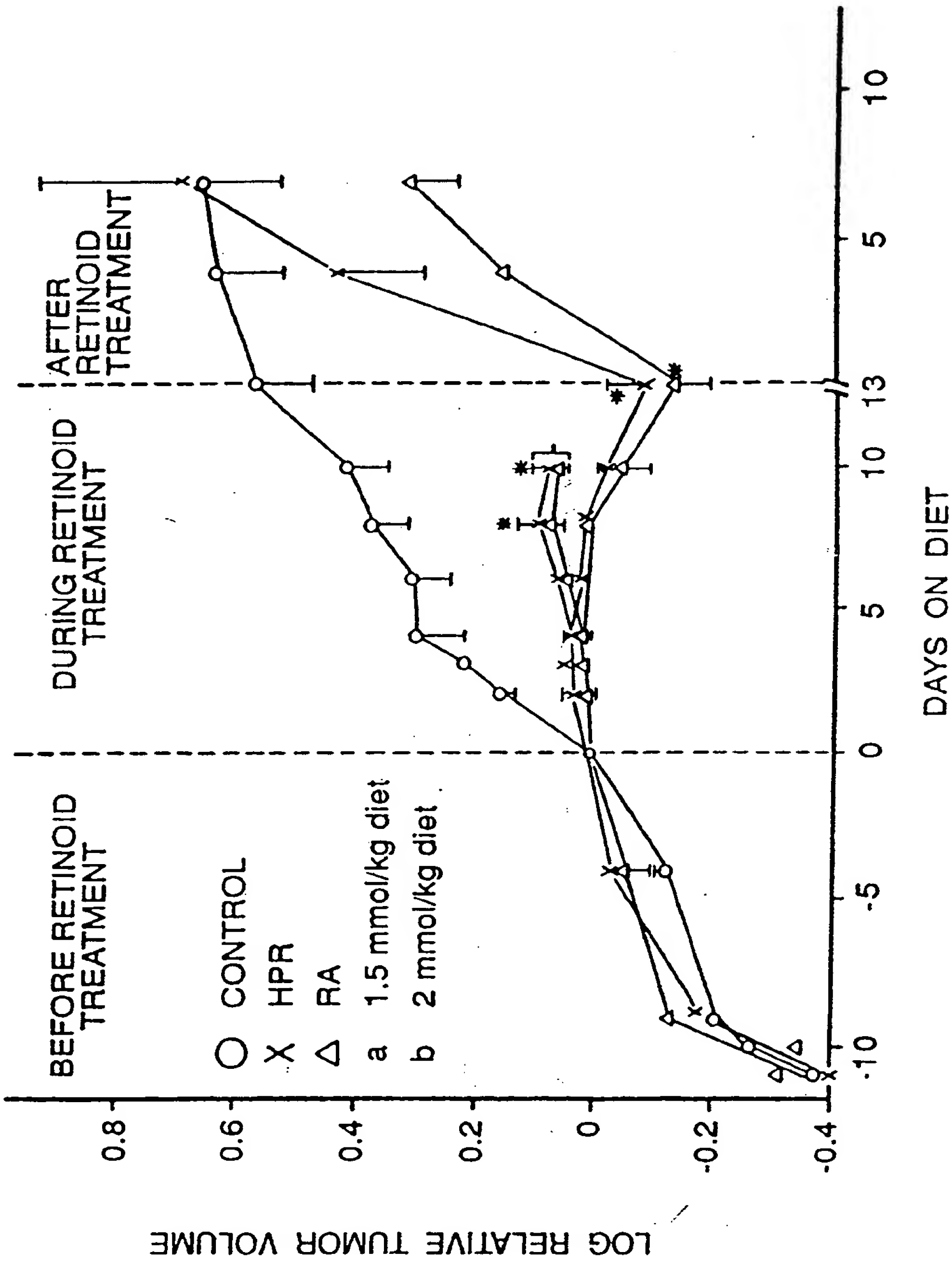
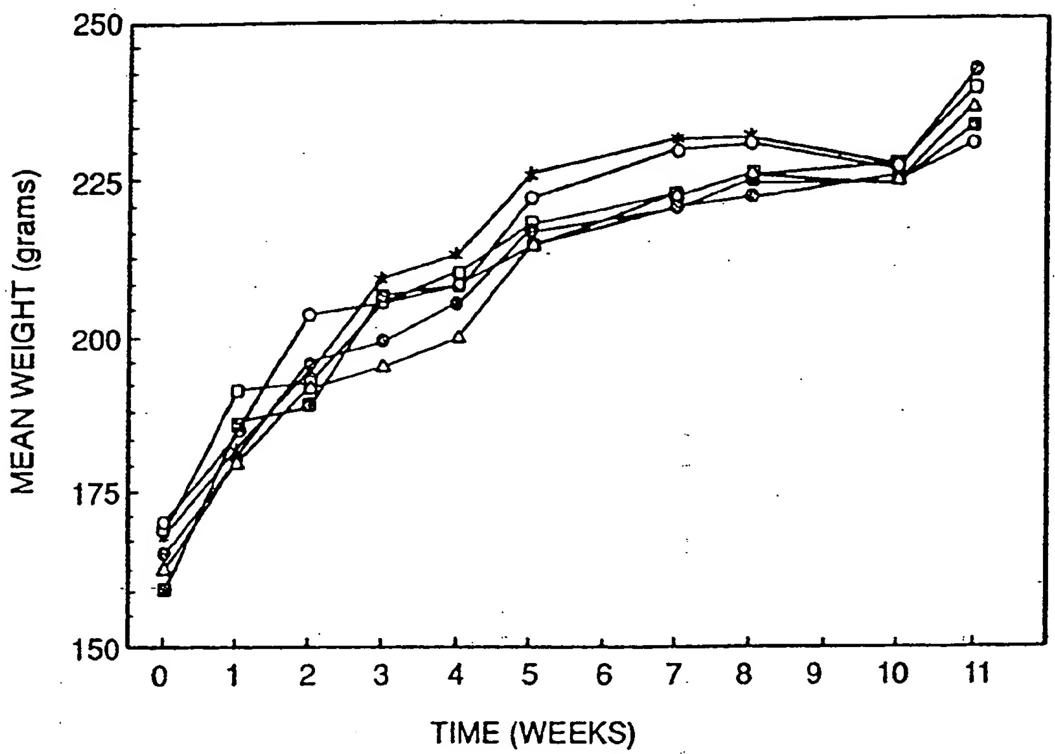

BEFORE RETINOID TREATMENT
DURING RETINOID TREATMENT
AFTER RETINOID TREATMENT
○ CONTROL
X HPR
△ RA
a 1.5 mmol/kg diet
b 2 mmol/kg diet
LOG RELATIVE TUMOR VOLUME
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-10
-5
0
5
10
13
5
10
DAYS ON DIET

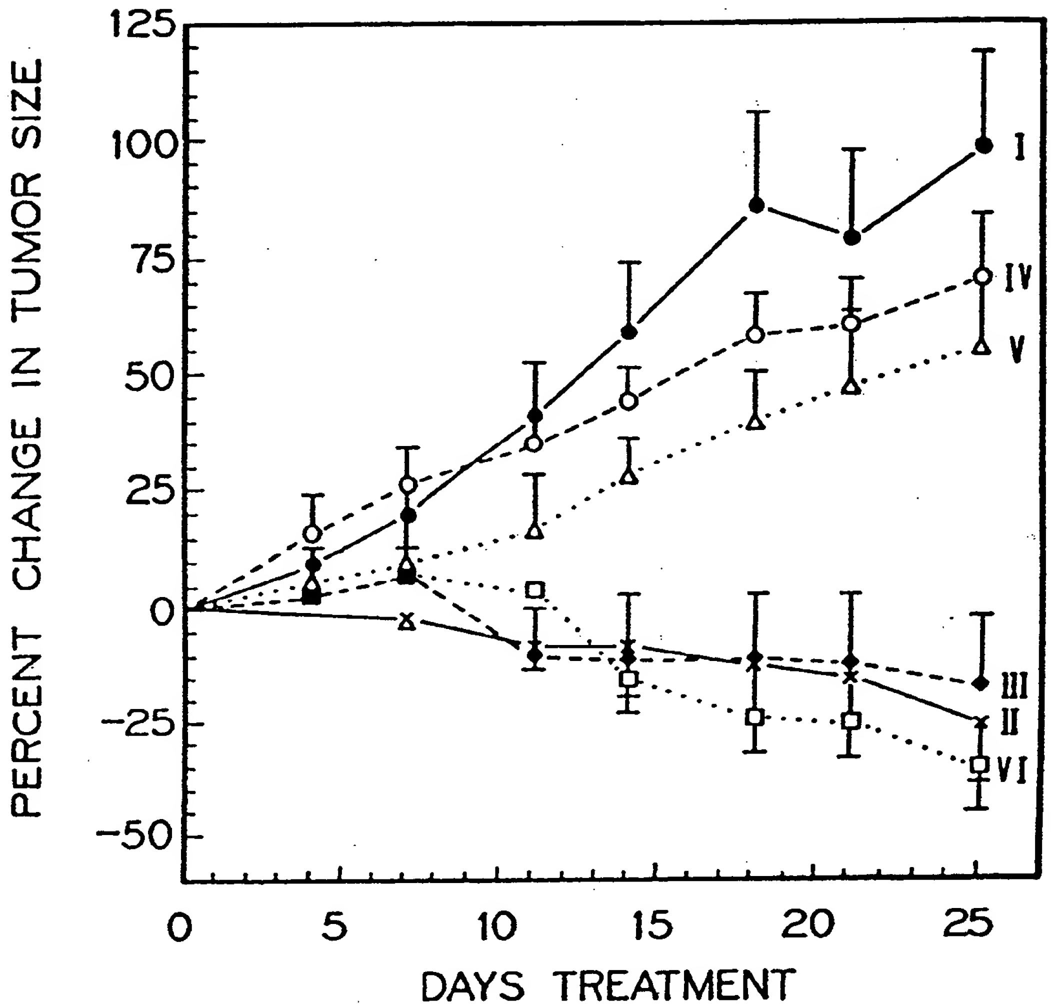
FIG. 3



EFFECT OF DIETS ON MEAN WEIGHTS OF RATS (gm)

- ✶ CONTROLS (CHOWDIET)
- ○ 1.0 % CGT
- ◎ 2.0 % CGT
- □ 0.75 mmol/kg HPR + 1 % CGT
- ▩ 0.75 mmol/kg HPR + 2 % CGT
- △ 1.5 % HPR

FIG. 4



125
100
75
50
25
0
−25
−50
PERCENT CHANGE IN TUMOR SIZE
0
5
10
15
20
25
DAYS TREATMENT
I
IV
V
III
II
VI

FIG. 5

METHOD AND COMPOSITION FOR ACHIEVING CHEMOTHERAPEUTIC ACTIVITY

The present invention is a continuation-in-part of patent application Ser. No. 089,568, filed Aug. 26, 1987, now abandoned.

This invention relates generally to a dietary supplement for achieving a very high degree of anticarcinogenic or chemopreventive activity and particularly relates to a combination of a low suboptimal doses of a D-glucarolactone-based dietary anticarcinogen with a low suboptimal doses of a retinoid-based anticarcinogen.

BACKGROUND OF THE INVENTION

Various glucarolactone-based compounds, including calcium glucarate (CGT), micro-encapsulated D-glucaro-1,4-lactone, potassium hydrogen glucarate and 2,4-di-O-acetyl-D-glucaro-1,4-lactone, are known to be effective as inhibitors of beta-glucuronidase in cells, blood, urine and in the intestine and liver. By inhibiting beta-glucuronidase, less detoxified (that is glucuronidated) toxins are hydrolysed and therefore more toxins are excreted. As a result, such glucarolactone-based compounds are useful in the treatment and prevention of various types of cancer.

Recently glucarate in the sustained release dietary form of calcium glucarate, was found to be a potent chemopreventative agent in the rodent system as having preventative activity against chemical carcinogenesis (both initiation and promotion phases) in the liver, Oredipe, O. A., et al., Effects of calcium glucarate on the promotion of diethylnitrosamine-initiated altered hepatic foci in rats in Cancer Letters 38: 95–99 (1987); lung, Walaszek, Z., et al., Dietary glucarate-mediated reduction of sensitivity of murine strains to chemical carcinogenesis in Cancer Letters 33: 25–32 (1986); skin, Dwivedi, C., et al., Modulation of chemically initiated and promoted skin tumorigenesis in CD-1 mice by dietary glucarate in *J. Environ. Pathol. Toxicol. and Oncol.*, in press; and mammary gland, Walaszek, Z., et al., Dietary glucarate as anti-promoter of 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis in Carcinogenesis, 7: 1463–1466 (1986). See, also for example, Walaszek, Z. et al. Inhibition of 7,12-dimethylbenzanthracene-induced rat mammary tumorigenesis by 2,5-di-0-acetyl-D-glucaro-1,4:6,3-dilactone, an in-vivo beta-glucuronidase inhibitor. Carcinogenesis 5: 767–772, (1984); and, Walaszek, Z., et al., Inhibition of N-methyl-N-nitrosourea-induced mammary tumorigenesis in the rat by a beta-glucuronidase inhibitor. IRCS Medical Science 14: 677–678, (1986).

In Walaszek et al., Carcinogenesis 7, supra, preliminary evidence was obtained that glucarate also inhibits and indeed causes regression of DMBA-induced rat mammary tumors. Glucarate is a normal body constituent, as it is the end product of glucuronic acid metabolism which is excreted in the urine. However, glucarate is present in blood and tissues at very low levels. Glucarate is non-toxic in dosages up to 10% in the diet as calcium glucarate and is effective at dietary levels of 4% (128 mmol/kg diet). In the systems studied to date it has been indicated that the active component is glucarate. Glucarate can undergo equilibrium formation of D-glucaro-1,4-lactone. Through inhibition of beta-glucuronidase, the lactone effects net formation of glucuronide conjugates of certain carcinogens or promoting agents in the phase II detoxification reactions, Dwivedi, C., et al., Net glucuronidation in different rat strains: Importance of microsomal beta-glucuronidase. The FASEB Journal, 1: 303–307 (1987).

Retinoid-based compounds including retinylacetate, retinylmethyl ether, 13-cis-retinoic acid and N-(4-hydroxyphenyl) retinamide (HPR), have similarly been investigated for their anticarcinogenic activity. These retinoids, both natural and synthetic, have been under intensive investigation as both cancer chemopreventative agents and anti-cancer (chemotherapeutic) agents, Moon, R. C., et al., Retinoids and cancer. In Sporn, M. B., Roberts, A. B., Goodman, D. S. eds., The retinoids, Vol 2. New York: Academic Press, 327–371 (1984) and Thompson, H. J., et al., Comparative review of the efficacy of a polyamine antimetabolite retinoids and selenium. J. Natl. Cancer Inst. 77: 595–598 (1986). Retinoids have been shown to be very effective at or near toxic levels in several experimental animal systems including MNU, Moon, R. C., et al., Retinylacetate inhibits mammary carcinogenesis induced by N-methyl-N-nitrosourea, Nature 267: 620–621 (1977); and the 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumor system, Abou-Issa, H., et al., Anticarcinogenic effect of retinoids on 7,12-dimethylbenz(a)anthracene-induced mammary tumor induction, and its relationship to cyclic AMP-dependent protein kinase, Biochem. Biophys. Res. Commun., 135: 116–123 (1986). Many of the toxicity problems were minimized in protocols associated with mammary carcinogenesis with the introduction of the synthetic retinoid N-(4-hydroxyphenyl)retinamide which displays trophism for the mammary gland, Moon, R. C., et al., N-4-Hydroxyphenyl retinamide, a new retinoid for preventing breast cancer in the rat, Cancer Res. 39: 1339–1349 (1979). This compound is currently being tested clinically in combination with the anti-estrogen Tamoxifen ®, in the prevention of breast cancer in women at risk, Rustin, G. J. S., et al., The potential use of retinoids in oncology (Meeting Report). Brit. J. Cancer 51: 443–445 (1985). [See also, Abou-Issa, H., et al., Anti-carcinogenic effect of retinoids on 7,12-dimethylbenz(a)anthracene-induced mammary tumor formation and its relation to cyclic AMP-dependent kinase Biochem. Biophys. Res. Commun. 135: 116–123, (1986); Welsch, C. W., et al., Retinoids and Mammary gland tumorigenesis in *Diet, Nutrition and Cancer* (B. S. Reddy and L. A. Cohen eds.) CRS Press Boca Raton, FL. pp 1–21, (1986); Schamberger, R. J. Chemoprevention of cancer in *Diet, Nutrition and Cancer.* (B. S. Reddy and L. A. Cohen eds.) CRC Press, pp. 43–62, (1986); and, Moon, R. C., Inhibition of 7,12-dimethylbenzanthracene-induced mammary carcinogenesis by retinyl acetate. Cancer Res. 36: 2626, (1976).]

These studies confirmed the activity of relatively high doses of retinoids against the chemical induction of mammary carcinogenesis in the rat. Similarly, high dosages were tested against the chemical carcinogen-mediated induction of tumors in the mammary gland, lung, skin, intestine and liver. Further, retinoids have been shown to protect skin, nasopharnyx, lower respiratory tract, urinary bladder and colon against carcinogens. In addition, these retinoic acid analogs (Vitamin A active compounds) have been tested in combination with the micronutrient selenium.

One problem associated with the use of retinoid-based compounds is that relatively high doses of the retinoids must be administered in order to achieve the

desired anticarcinogen effect. Such high doses of retinoids often results in cummulative toxicity, with the excess retinoids being deposited in the liver.

Because relatively high levels of around 4% of dietary calcium glucarate are required for maximum effectiveness, and because of potential retinoid toxicity and the desire to increase the therapeutic index, the combination of the retinoid and glucarate was disclosed in the parent patent application Ser. No. 089,568. Surprisingly, when low ineffective dosages (approximately one-half the effective dose or less) of HPR and CGT were combined in the diet, they interacted synergistically to inhibit carcinogenesis to the extent of high effective dosages of either agent alone. The results of this patent application were later reported in Abou-Issa, H. M., et al., Putative metabolites derived from dietary combinations of calcium glucarate and N-(4-hydroxyphenyl)retinamide act synergistically to inhibit the induction of rat mammary tumors by 7,12-dimethylbenz-(a)anthracene. Proc. Natl. Acad. Sci. (U.S.) 85: 4181–4184 (1988). This combination has now been further evaluated as a chemotherapeutic agent on established DMBA-induced rat mammary tumors. The results disclosed herein indicate that the combination of the retinoid and the glucarate also interact synergistically to inhibit rat mammary tumor growth.

There has been no suggestion in the art that a combination of glucarolactone-based compounds and retinoid-based compounds would be especially useful as anticarcinogens; that is, that the combination of these compounds would represent an alternative for use in the prevention of cancer or for use in the therapeutic treatment of cancer.

It has now been found that the use of a combination of glucarolactone-based compounds and retinoid-based compounds or their pharmaceutically-acceptable salts and esters, compounds which are known to be safely administered to humans and animals, significantly inhibit tumor growth and to shrink tumors.

It is therefore an object of the present invention to provide a safe and effective dietary supplement and method for inhibiting tumor growth and for reducing the incidence of cancer in certain high risk populations.

Other objects and advantages of the invention will be apparent from the following detailed description of the invention.

SUMMARY OF THE INVENTION

The present invention describes a protocol for achieving a very high degree of chemotherapeutic activity through the combination of a low suboptimal dose of a D-glucarolactone-based dietary anticarcinogen (namely, calcium glucarate) with a low suboptimal dose of retinoid-based anticarcinogen (namely, 4-hydroxyphenyl retinamide). Since synergism is obtained by this combination, these two anticarcinogens can be employed at dosages far below toxic levels and at dosages that are practical as a chemotherapeutic agent.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a graph showing the effect of diets on the mean number of tumors per rat: •control (chow diet); Δ 1.5% HPR; □ 0.75 mmol/kg HPR and 1% CGT; and, 0.75 mmol/kg HPR and 2% CGT.

FIG. 2 is a graph showing the effect of diets on the percentage of rats with tumors: •control (chow diet); 1% CGT; 2% CGT; □ 0.75 mmol/kg HPR and 1% CGT; 0.75 mmol/kg HPR and 2% CGT; Δ 1.5 mmol/kg HPR; and 0.75 mmol/kg HPR.

FIG. 3 is a graph showing the effects of retinoids on inhibiting growth of established cancer, before retinoid treatment, during retinoid treatment and after retinoid withdrawal: control; X HPR; Δ RA; (a) 1.5 mmol/kg diet, (b) 2 mmol/kg diet.

FIG. 4 is a graph showing the effect of diets on the mean weight of rats: •control (chow diet); 1.0% CGT; 2.0% CGT; □ 0.75 mmol/kg HPR and 1% CGT; 0.75 mmol/kg HPR and 2% CGT; and Δ 1.5% HPR.

FIG. 5 is a graph showing the time-course change in volume of mammary tumors as a function of time (days) on the following diets, (group; dosage in mmol/kg diet; symbol): I, chow ( ); II, chow+2.0 mmol/kg HPR (X); III, chow+128 mmol/kg CGT ( ); IV, chow+0.75 mmol/kg HPR ( ); V, chow+64 mmol/kg CGT (Δ); VI, chow+0.75 mmol/kg HPR+64 mmol/kg CGT (□).

DESCRIPTION OF INVENTION

The present invention relates to a method for achieving a very high degree of chemotherapeutic activity comprising the administration of a safe and effective amount of a compound comprising a combination of a D-glucarolactone-based dietary anticarcinogen with a retinoid-based anticarcinogen and pharmaceutically-acceptable salts and esters thereof to a subject either in a high risk group for cancer or to a subject who has cancer.

The treatment regimens encompassed by the present invention employ a safe and effective amount of a pharmaceutically-acceptable composition comprising a combination of a glucaro-lactone-based and a retinoid-based compound. These compounds are administered to prevent the occurrence of cancer and to inhibit the growth of cancer tumor cells in humans and animals. Various glucarolactone-based compounds utilized herein are conveniently abbreviated "glucarolactone" or "GL"; "calcium glucarate" or "CGT"; various retinoid-based compounds utilized herein are conveniently abbreviated "retinoids" or "HPR". The phrase "safe and effective amount of glucarolactone/retinoid compound" herein, means sufficient glucarolactone/retinoid compound to desirably affect and inhibit the induction or growth of tumor cells, at a reasonable benefit/risk ratio attendant with any medical treatment. Within the scope of sound medical judgment, the required dosage of the glucarolactone/retinoid compound will vary with the severity of the condition being treated, the duration of the treatment, the nature of adjunct treatment, the age and physical condition of the patient, the specific glucarolactone and retinoid compounds employed, and like considerations discussed more fully hereafter.

"Pharmaceutically acceptable", as used herein, means that the glucarolactone/retinoid compound and other ingredients used in the compositions employed herein are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio.

The term "administration" of the glucarolactone/retinoid compounds and compositions, as used herein includes intragastric and oral administration thereof.

The term "comprising", as used herein, means that various other compatible drugs and medicaments, as

well as inert ingredients, can be conjointly employed in the therapeutic methods of this invention, as long as the critical glucarolactone/retinoid compounds are used in the manner disclosed. The term "comprising" thus encompasses and includes the more restrictive terms "consisting of" and "consisting essentially of".

By "compatible", herein, it is meant that the components of the composition used in the practice of this invention are capable of being comingled without interacting in a manner which would substantially decrease the efficacy of the glucarolactone/retinoid compositions under ordinary use situations.

The novel compositions of the invention are useful for the treatment of various cancers, such as for example, lung, colon and mammary cancers. The novel compositions may be used alone or in combination with other therapeutic agents active for these purposes. As used herein, the term "inhibition" comprehends arresting or retarding the growth of the malignancy or other manifestation of a disease, as compared with the course of the disease in the absence of treatment.

The novel compositions of the present invention are also useful to prevent the occurence of cancer in high risk populations. The compositions may be used alone or in combination with other chemopreventative agents active for these purposes. As used herein, the term "prevention" comprehends reducing the incidence of tumors in a patient or a population exposed to cancer causing agents such as cigarette smoking, or environmental toxins, as compared with the course of potential development of this disease in the absence of treatment. It also comprehends reducing endogenously produced cancer-causing agents including steroid hormones.

The mechanism of action of retinoid-based compounds such as retinylacetate, retinylmethyl ether, 13-cis-retinoic acid, and N-(4-hydroxyphenyl)retinamide (HPR) are believed to act by inducing differentiation. One of the main biochemical effects of the retinoid-based compound is to elevate the level of cellular cAMP (cyclic AMP) and of histone kinases.

Similarly, the glucarolactone-based compounds such as calcium glucarate, potassium hydrogen glucarate, micro-encapsulated D-glucaro-1,4-lactone, 2,4-di-O-acetyl-D-glucaro-1,4-lactone, when fed slowly release D-glucaro-1,4-lactone (GL), a potent inhibitor of beta-glucuronidase in the cells, blood and urine and in the intestine. Since beta-glucuronidase is inhibited, less detoxified (i.e., glucuronidated) toxins are hydrolyzed and therefore more toxins are excreted. Thus, the inhibition of beta-glucuronidase promotes clearance/excretion of detoxified (glucuronidated) compounds from the body. Not only carcinogens but other toxins, steroid hormones and other substances which undergo glucuronidation may be affected.

The above-mentioned compounds are used as dietary sources of GL since they are more effective by virtue of the fact they are sustained (slow)-release forms, GL itself being too rapidly absorbed and cleared from the body. Thus, the glucarolactone-class of inhibitors may be used to reduce the inappropriate level of any compound in the body which is subject to glucuronidation before excretion. Besides the glucarates, micro-encapsulated GL and the di-O-acetyl derivative of GL, dietary substances which may yield GL and which might be as useful as CGT include D-glucuronic acid, D-galacturic acid, L-iduric acid or derivatives or analogs thereof.

Although CGT in one embodiment of the invention is combined with retinoids it is also possible that CGT may be effectively combined with other micronutrients or even lower doses of CGT and retinoid may be combined with additional anticarcinogens. For example, calcium appears to be an anticarcinogen for colon cancer by ameliorating the toxic effects of bile acids so that the calcium glucarate/retinoid combination may be considered to be a combination of three anticarcinogens, though the protective effect of these low dosages of calcium are minimal.

A particularly interesting combination might be CGT/retinoid/ascorbic acid, since Vitamin C is protective against colon cancer. Because of activity against carcinogens attacking most major organs, whereas other anticarcinogens are more organ-specific, the glucarolactone-based/retinoid-based anticarcinogens can serve as a common component in combinations with other known anticarcinogens.

A combination of low non-toxic doses of dietary retinoid (HPR) and dietary calcium glucarate inhibited the incidence of dimethyl benz(a)anthracene-induced rat mammary tumors to a greater extent than the same doses of either agent alone. This combination was also able to reduce the number of palpable tumors by one-half (50%) as compared to rats that received the control diet, or identical doses of either agent alone. These results, which are relevant to breast cancer, may also apply to chemoprevention of cancer at other sites, including higher doses of CGT which have been shown to be effective against lung, colon and mammary carcinogenesis while retinoids are known to be effective against at least mammary and colon carcinogenesis.

A combination of suboptimal doses of retinoid and glucarolactone-based compounds is effective as an anti-carcinogen. Furthermore, by reducing the dosage of the anti-cancer agents, both toxicity and impractical dosage requirements are circumvented.

EXAMPLE I

The following example demonstrates the heretofore unsuspected ability of the composition of the present invention to desirably inhibit the formation of tumors. The effect of CGT, HPR and CGT/HPR on 7,12-dimethylbenz(a)anthracene(DMBA)-induced mammary tumor formation in female Sprague Dawley rats was determined using the following protocol: Female rats maintained on one of 8 diets received 75 mg/kg of 7,12-dimethylbenz(a)anthracene in mineral oil by mouth. They were maintained on the diets for approximately 4 mos., and were examined (palpated) for mammary tumors weekly. The diets were (i) rat chow; (ii) chow and 1% CGT; (iii) chow and 2% CGT; (iv) chow and 4% CGT; (v) chow and 0.75 mmol/kg HPR; (vi) chow and 1.5 mmol/kg HPR; (vii) chow and 1% CGT and 0.75 mmol/kg HPR; and (viii) chow and 2% CGT and 0.75 mmol/kg HPR. Note: mmol/kg means mmol/kg diet; 1% CGT means 1 gm/100 gm of chow. In these experiments the CGT powder was mixed into the powdered chow diet. The retinoid (HPR) was first dissolved in 25 ml of a vehicle consisting of ethanol-tricaprylin-6% α-tocopherol, then thoroughly mixed with powdered rat chow. The results obtained, expressed in tumor incidence, total number of tumors, and tumors per rat, are summarized in the following Table I:

TABLE I

Effect of CGT, HPR and CGT & HPR on 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumor formation in Female Sprague Dawley Rats					
Dietary Anti-carcinogen	No. of Rats	Rats with Tumors	Tumor Incidence	Total No. Tumors	Tumors Per Rat
None (control)	20	14	70	22	1.1
1% CGT	20	11	55	20	1.0
2% CGT	20	11	55	20	1.0
4% CGT	20	7	35	10	0.5
0.75 mmol/kg HPR	20	12	60	20	1.0
1.5 mmol/kg HPR	20	6	30	7	0.35
0.75 mmol/kg HPR & 1% CGT	20	9	45	12	0.6
0.75 mmol/kg HPR & 2% CGT	20	7	35	9	0.45

% CGT = gm % of calcium glucarate added to chow diet. mmol/kg HPR = moles of 4-hydroxy phenylretinamine added to chow diet per kg diet.
Protocol: 50d old female S.D. rats received a single dose 75 mg/kg of DMBA. Feeding of CGT, HPR or both was initiated 2 weeks before treatment with DMBA, then continued throughout the experiment.

When tested alone the higher doses of CGT (4%) and HPR (1.5 mmol) markedly inhibited tumorigenesis i.e., tumor incidence by 50-60% and tumors/rat by 50-65%. At lower doses the effect was minimal i.e., 1.0% CGT inhibited tumor incidence only 20% and tumors/rat by 9% while 0.75 mmol/kg HPR inhibited tumor incidence only 15% and tumors/rat by 9%. In contrast, when tested in combination, 1% CGT and 0.75 mmol/kg HPR inhibited tumor incidence by 36% and tumors/rat by 45%. Similarly 2% CGT and 0.75 mmol/kg HPR inhibited tumor incidence by 50% and tumors/rat by 60%.

The effect of the diets on the mean number of tumors per rat is shown in FIG. 1, while FIG. 2 shows the effect of the diets on the percent of rats with tumors.

Glucarolactone-based inhibitors acts to inhibit the growth of hormone-dependent tumors by lowering hormone levels. It has previously been shown in Walaszek, Z., et al., Carcinogenesis 7: 1463-1466 (1986), that dietary calcium glucarate (CGT) inhibits the promotion phase of 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis. The female rats were put on the CGT diet two weeks after treatment with carcinogen. By 28 weeks tumor induction in the rats on the 4% CGT diet was only 30% of that in the controls, indicating CGT markedly inhibits the promotion phase in this model. Rats on the CGT-supplemented diet ate quantities of food and had weight gain identical to those on the normal chow diet. The anti-promotional effect of CGT was shown to be probably due to the reduction in the steady-state level of sex hormones. Further, and of relevance to this invention, some tumors on the chow diet supplemented with CGT underwent regression. The overall tumor incidence represents those which escape the anti-promotional effects of CGT and the equilibrium between growth and regression.

Similarly, retinoids, as shown in FIG. 3, inhibit growth of established cancer. The administration of 1.0 mmol/kg diet of HPR daily to female Sprague Dawley rats with already established DMBA-induced mammary tumors resulted in 80-90% inhibition of tumor growth within 10 days. Similarly, when given to CD_8F_1 mice with established mammary tumors this retinoid resulted in 50% inhibition of tumor growth. When higher doses (2 mmol/kg diet) of HPR were used, growth arrest was followed within 5 to 10 days by 30% regression of the DMBA-induced mammary tumors. Also, HPR (0.1 uM) inhibited the in vivo growth of the human breast cancer cell line (MCF-7) to 50% of the control within 7 days. These results suggest that retinoids have anti-tumor effects besides their cancer chemopreventive effects.

As shown in FIG. 4 prolonged feeding of diets containing CGT and HPR did not affect weight gain of rats. This is important since toxicity as evidenced by marked weight loss may also influence carcinogenesis and tumorigenesis.

The effect glucarolactone (GL) alone or in combination with HPR on the growth of MCF-7 cells is shown in Table II below. The cells were plated at the density of 3775 cc per well in six well plates and treated after one day with GL-HPR- or GL and HPR-containing media for 5 days. The values shown are the mean +/− ISE for three experiments counted in triplicates. The numbers in parenthesis indicate the number of free floating (mostly non viable) cells in the media. As can be seen, the GL/HPR-containing media had only one-third the viable cells as the control medium. The GL (10^{-4}M)/HPR(5×10^{-8}M)-containing medium had nearly one-third the viable cells as the GL (10^{-4}M) medium alone and had nearly one-half the viable cells as the HPR(5×10^{-8}M) medium alone. These experiments indicate that the combination of GL and HPR is capable of inhibiting tumor cell growth and that such combination is more effective than either the GL- or HPR-containing media alone in inhibiting tumor cell growth.

TABLE II

Treatment	No. of viable cells	% of control
0.1% Ethanal	60400 ± 2147 (6440)	100 ± 4 (11)
GL (10^{-4}M)	49480 + 3160 (3440)	82 + 5 (6)
GL (10^{-3}M)	32560 ± 1800 (4560)	54 ± 3 (7)
HPR (5×10^{-8}M)	33680 ± 2627 (4160)	56 ± 4 (7)
GL (10^{-4}M) + HPR (5×10^{-8}M)	18160 ± 1568 (5280)	30 ± (9)
GL (10^{-3}M) + HPR (5×10^{-8}M)	22680 ± 1863 (2960)	37 ± 3 (5)

EXAMPLE II

The following example demonstrates the heretofore unsuspected ability of the composition of the present invention to not only desirably inhibit tumor growth but also shrink tumors. Rat mammary tumors were induced by treating one hundred 50 day old female Sprague Dawley rats (Harlan Industries, Indianapolis, Ind.) by gavage with a single dose of 15 mg of 7,12-dimethylbenz(a)anthracene (Sigma Chemical Co., St. Louis, Mo.) in 1.0 ml of sesame oil, Abou-Issa et al.,

Proc. Natl. Acad. Sci. U.S.A., supra. The rats were maintained on a chow diet ad libitum until the tumors had formed in 70% of the rats and had reached a size of approximately 2.0 cm in diameter; 4 months later at which time they were randomized into six groups of 10 rats each. Tumor sizes were measured in all the rats just before placing them on the following experimental diets (Groups I-VI): (I) rat chow (RMH 3200, Pro Lab, Syracuse, N.Y.); (II) rat chow with HPR (1.5 mmol/kg); (III) rat chow with 4% CGT 3.5 H_2O Gallard Schlesinger, Carle Place, N.Y.) (128 mmol/kg of diet); (IV) rat chow with HPR (0.75 mmol/kg); (V) rat chow with 2% CGT (64 mmol/kg); (VI) rat chow with the combination 2% CGT (64 mmol/kg) and HPR (0.75 mmol/kg). The CGT was added as a powder directly to the diet, while the HPR was first dissolved in 25 ml of ethanol/tricaprylin 1:4 (vol/vol) plus 6% (wt/vol) α-tocopherol, as previously described in Abou-Issa et al., *Proc. Natl. Acad. Sci. U.S.A.*, supra. The latter vehicle was added to the diet of all six groups. The additives were blended into the diet with a mechanical mixer.

All the tumors that developed within the time frame of this example are histologically adenocarcinomas. This finding has been previously reported by Huggins et al., Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression, Nature, 185:204–207 (1961), in this animal model under the same conditions, and has been confirmed in several indentical experiments. At later time periods beyond the time frame of this example fibroadenomas usually develop, Welsch et al., In "Cellular and Molecular Biology of Mammary Cancer", (W. Kidwell, G. Heppner and E. Anderson, eds.) Plenum Press, N.Y. 1987, pp 163–179. Tumor measurements were began at the time the rats were placed on the experimental diets and were continued twice weekly throughout the duration of the experiment. Two axes were measured - the longest axis (L) and the axis at right angles to the longest (W), using a vernier caliper. Tumor volume was calculated from the formula 4/3 pi r^3, where r is half the mean of the two diameters in accordance with Steel, Growth kinetics of tumors, Oxford Univ. Press, London, pp 5–25, 1977.

Tumor regression is indicated by at least 20% reduction in tumor size and each tumor is compared to its initial size on day zero. Weights of the rats on the control and experimental diets were recorded weekly.

Estrogen and progesterone receptors were measured in the OSU Hormone Receptor Laboratory by the multipoint dextran-coated charcoal method as previously described in Schuller et al., Estrogen and progesterone receptors in head and neck cancer, Arch. Otolaryng. 110:725–727 (1984). The data of specific binding were analyzed by Scatchard, The Attraction of proteins for small molecules and ions, Ann. N.Y. Acad. Sci. 51:660–672 (1949), plots to determine the equilibrium dissociation constant (K_d) and binding capacity expressed as femtomoles of estradiol or progesterone (R-5020) specifically bound per mg of cytosol protein for estrogen and progesterone receptors respectively. Cytosol protein concentrations were determined by the procedure of Lowry et al., Protein measurement with the folin phenol reagent, J. Biol. Chem. 193:265–275 (1951).

FIG. 5 is a graph showing the effect of the six diets on tumor growth. When tested separately (groups II and III), optimal doses of HPR (2 mmol/kg) or CGT (128 mmol/kg) decreased mammary tumor sizes by 15% and 25%, respectively. Tumors continued to grow in rats fed on the chow diets and on diets supplemented with low ineffective doses of calcium glucarate (group IV) or N-(4-hydroxyphenyl) retinamide (group V). At the low dose of HPR (0.75 mmol/kg) the size of the tumors increased by 70% compared to 98% in the controls. Similarly, at low dose CGT (64 mmol/kg) enhanced tumor growth, the size of the tumors increased by 55% over the 25 day period of observation. In contrast, the combination of CGT+HPR (group VI) had a marked synergistic inhibitory effect on rat mammary tumor growth over the 25 day period of observation. In this instance tumors decreased in size by 33% compared to the initial size at the beginning of the treatment.

The tumor measurements, being positively skewed, were log transformed in order to more accurately satisfy the assumptions of the analysis of variance. A repeated measures analysis of variance (with one between animal factor, treatment, and one repeat factor, time) was performed on the transformed data using baseline tumor size as a covariate. There was a statistically significant group by time interaction ($p<0.0001$) which indicates that the profile of tumor size over time depends upon group membership (See FIG. 5).

Upon employing follow-up Tukey multiple comparison tests (overall $\alpha=0.05$), adjusting for baseline tumor size, the following results were obtained. No statistically significant differences for the groups were discovered for the first three time periods (days 4, 7 and 11). At the fourth time period (day 14), groups II and III showed significantly decreased tumor sizes compared to the control group (group I). Also group III showed decreased tumor sizes compared to group IV. For the last three time periods (days 18, 21 and 25), groups I, III, and VI showed significantly decreased tumor sizes compared to the control group (group I), as well as to group IV and V. Although there is the potential for some error in measuring tumor size, the variability of the random error, i.e., the amount of error that could not be explained by group membership and days on treatment, was small, compared to the group difference at the latter time periods referred to above as being statistically significant.

It is known that weight gain/food intake may affect tumor growth as reported in Klurfeld et al., Determination of degree of energy restriction necessary to reduce DMBA-induced mammary tumorigenesis in rats during the promotion phase, J. Nutr. 119:286–291 (1989). Therefore, rats in all groups were weighed weekly and the results are shown in Table III below. Weight gain was essentially identical in all groups.

TABLE III

Average Weight of the Rats Just Before Treatment and at the End of Experiment

Treatment	Initial Weight (gm)	Final Weight (gm)
Control (Chow + Vehicle)	418 +/− 28	437 +/− 30
HPR (2.0 mmol/kg)	401 +/− 13	413 +/− 10
CGT (4%)	404 +/− 22	429 +/− 26
HPR (0.75 mmol/kg)	409 +/− 18	421 +/− 14
CGT (2%)	401 +/− 10	415 +/− 15
HPR (0.75) + CGT (2%)	418 +/− 15	428 +/− 18

The mechanism underlying the synergistic interaction of HPR and CGT is not clear. However, effective levels of glucarate are known to slightly reduce the level of circulating steroid hormones, including estrogen or testosterone (Walaszek et al., Carcinogenesis, 7, supra, possibly through phase II reactions, but it is ques-

tionable whether low levels act similarly. Effective doses of retinoid induces differentiation in some tumor cell histio-types, but again the effect at the lower dosages is unclear. To obtain some indication of mechanism, the estrogen receptor levels were measured in the tumors remaining in each group at the termination of the experiment. As shown in Table IV below, there is no significant difference in the estrogen/progesterone receptor profile in the group receiving the combination compared to the other groups.

TABLE IV

Effect of HPR and CGT on the Estrogen and Progesterone Receptors of the Established DMBA-Induced Mammary Tumors

Treatment	N	Estrogen Receptors (Fmoles/mg Protein)	Progesterone Receptors (Fmoles/mg Protein)
Control (chow + vehicle)	7	46.7 +/− 8.9	96.6 +/− 13.9
HPR (2.0 mmol/kg)	7	50.6 +/− 8.4	89.3 +/− 13.7
CGT (4%)	7	44.3 +/− 5.9	82.0 +/− 16.0
HPR (0.75 mmol/kg)	7	47.0 −/− 8.5	90.7 +/− 18.0
CGT (2%)	7	42.7 +/− 7.0	84.4 +/− 17.0
HPR (0.75) + CGT (2%)	7	51.7 +/− 9.0	96.3 +/− 29.0

When fed at low ineffective dosages, CGT and HPR had no observable inhibitory effect and indeed enhanced tumor development when fed separately. However, in combination they inhibited tumor growth by over 33% over a four week period. Furthermore, the combination caused tumor regression in greater than 80% of the rats, a very significant effect. These data are relevant where a drug is considered active in any particular tumor histio-type if it yields positive in at least 70% of the patients, Stolfi et al, Chemotherapeutic evaluation using clinical criteria in spontaneous autochthonous murine breat tumors, J. Natl. Cancer Inst. 80:52–55 (1988).

One possibility of the mechanism of action of the synergistic combination is that through conversion to D-glucaro-1,4-lactone and consequent inhibition of beta-glucuronidase, the net formation of HPR-glucuronide is enhanced. The D-glucaro-1,4-lactone compound is believed to be more effective and less toxic than the parent compound. Retinoid-glucuronides have previously been shown to have efficacy in tumor cell culture system, Zile et al., Induction of differentiation of human promyelocytic leukemia cell line HL-60 by retinoyl glucuronide, a biologically active metabolite of vitamin A, Proc. Natl. Acad, Sci. (U.S.A.) 84:2208–2212 (1987); Gallup et al., Effects of retinoid beta-glucuronides and N-retinoylamines on differentiation of HL-60 cells in vitro, Proc. Soc. Exp. Biol. Med. 186:269–294 (1987).

It is shown herein that combinations of low ineffective doses of HPR and CGT give a synergistic antitumor (chemotherapeutic) activity which is highly effective against rat mammary carcinoma.

It may be advantageous to formulate the compositions of the invention in unit dosage form for ease of administration and uniformity of dosage. A unit dosage formed, as used herein, refers to a physically discrete unit suitable for use as a unitary dosage for the mammalian subjects to be treated; each unit contains a predetermined quanitity of active material calculated to produce the desired therapeutic effect, in association with the required pharmaceutically acceptable carrier. Specifications for unit dosage forms are dictated by and directly dependent on (a) the unique characteristics of the active material in the particular therapeutic affect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition, without excessive cytotoxic effects.

Regression of breast cancer and inhibition of tumor growth may be obtained, for example, by the use of daily dosing for up to 50 to 100 days, or longer. Multiple dosing, or dosing on any desired periodic basis, may also be utilized. The therapeutically active ingredient is thus administered in an amount sufficient to aid regression and inhibition of further growth of the cancer, in the absence of excessive deleterious side-effects of a phyto-toxic nature.

The minimum dosage of the anticarcinogens used alone, consistent with maximum inhibition of carcinogenesis is approximately equivalent to 4 gm % CGT (4 gm/100 gm of chow diet) or 1.0–1.5 mmol/kg of HPR. This consideration is important since the human (in proportion to the surface area of the rodent) would need to consume approximately 40 gm of CGT per day while a dosage of 1.5 mmol/kg of retinoid would result in cummulative toxicity, with excess being deposited in the liver. Thus previous studies have been hindered by concern and actual problems with the toxicity of effective doses of the retinoids. The problems common to the use of these two classes of anticarcinogens when used separately, is circumvented by their combination. Furthermore, since their efficacy was tested using a carcinogenic protocol which utilized a single high dose of carcinogen (the minimum effective dose), it may be possible to reduce their concentrations even lower under chronic dose exposure to carcinogens. Thus, we have found that a combination of 2% CGT and 0.75 mmol/kg of retinoid is as effective as the single higher doses of each.

The anticarcinogenic combination of CGT and HPR compounds (active ingredients) of this invention can be administered to inhibit the formation of tumor cells or to decrease the risk of contracting cancer by any means that produces contact of the active ingredient with the agents site of action in the body of a human or animal. This anti-carcinogenic combination can also be administered to inhibit tumor growth and cause tumor regression. The combination can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or a combination of therapeutic active ingredients. The combination can be administered along, but is generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will be a tumor-inhibiting amount of active ingredient and will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular active ingredient, and its mode and route of administration; age, health, and weight of receipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effects desired. Usually a daily dosage of active ingredient can be about 5–400 mg/kg of body weight. Ordinarily, 10–300, and preferably 100–300 mg/kg body weight per day given in single doses or divided doses 2–4 times a day or in sustained release form is effect to obtain desired results. In a preferred embodiment the dietary supplement comprises approximately 0.01 to 0.02 parts by weight of the glucarolactone-based compounds for inhibiting beta-glucuronidase and approximately 0.0003 to 0.0006 parts

by weight of the retinoid-based compound for elevating levels of cellular cAMP and of histone kinases.

Dosage forms (compositions) suitable for internal administration contain from about 1.0 mg to about 500 mg of active ingredient per unit. In these pharmaceutical compositions active ingredient will normally be present in an amount of 0.5-95%, by weight, based on the total weight of the composition.

The active ingredient can be administered in the diet or in solid dosage forms such as capsules, tablets and powders or in liquid dosage form, such as elixers, syrups and suspensions.

Gelatin capsules contain the active ingredient and powdered carriers, such as lactose, sucrose, mannitol, starch, cellulose derivatives, magnesium stearate, stearic acid, and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of the composition over a period of hours. Compressed tablets can be sugar-coated or film-coated to mask any unpleasant taste and protect the tablet from atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract.

Liquid dosage forms for oral administration can contain coloring and flavor to increase patient acceptance.

Useful pharmaceutical-dosage forms for administration of the compounds of this invention can be illustrated as follows:

CAPSULES: a large number of unit capsules are prepared by filling standard two-piece hard gelatin capsules each with 100 mg of powdered active ingredient, 175 mg of lactose, 24 mg of talc, and 6 mg magnesium stearate. A mixture of active ingredient in soybean oil is prepared and injected by means of a positive displacement pump into gelatin to form soft gelatin capsules containing 100 mg active ingredient. The capsules are washed and dried.

TABLETS: Large number of tablets are prepared by conventional procedures so that the dosage unit is 100 mg of active ingredient, 0.2 mg of colloidal silicon dioxide, 5 mg of magnesium stearate, 275 mg of microcrystalline cellulose, 11 mg of cornstarch and 98.8 mg of lactose. Appropriate coatings may be applied to increase pallatability or delayed absorption.

For treatment of non-human animals, the composition is preferably incorporated in animal feed, feed supplements or feed concentrates.

From the preceding, it can be seen that in accordance with the present invention, a novel composition comprising glucarolactone-based compounds and retinoid-based compounds is provided, the members of the compositions of which induce regression and/or inhibit the induction and growth of various malignant tumors in mammals.

It will be apparent that various changes may be made in the method of preparation and use, as well as in the particular substitution of therapeutically active compositions of the present invention. Accordingly, the preceding disclosure should be construed as illustrative only, and the scope of the claims should be incorporated in accordance with the claims appended hereto.

We claim:

1. A dietary supplement for use as a chemotherapeutic agent in treating mammary cancer in humans or animals via steady and prolonged inhibition of beta-glucuronidase and via elevation of the level of cellular cAMP (cyclic AMP) and of histone kinases, the supplement consisting essentially of approximately 0.01 to 0.02 parts by weight of calcium glucarate for inhibiting beta-glucuronidase and approximately 0.0003 to 0.0006 parts by weight of N-(4-hydroxyphenyl) retinamide for elevating levels of cellular cAMP and of histone kinases.

2. The dietary supplement of claim 1, wherein a daily dosage of the supplement is from about 100 to about 300 mg/kg body weight.

3. The dietary supplement of claim 1 wherein the supplement is administered promptly after the detection of the tumor.

4. A method of treating mammary cancer in humans or animals comprising administering an amount, which is safe and sufficient of the dietary supplement of claim 1 including the glucarolactone-based compound and the retinoid-based compound, or a pharmaceutically acceptable salt thereof to a patient having at least one mammary tumor.

5. The method of claim 4, wherein a daily dosage of the supplement is from about 100 to about 300 mg/kg body weight.

6. The method according to claim 4, wherein the supplement is administered promptly after the detection of the tumor.

7. A pharmaceutical composition for use as a chemotherapeutic agent for treating mammary cancer in humans or animals which comprises a therapeutically effective amount of the supplement of claim 1 in admixture with a pharmaceutically acceptable, substantially non-toxic carrier or excipient.

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[54] COMBINED DEHYDROEPIANDROSTERONE AND RETINOID THERAPY FOR EPITHELIAL DISORDERS

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[52] U.S. Cl. 514/171

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[57] ABSTRACT

The present invention relates to compositions and methods for the treatment of oxidative epithelial damage, for inadequate surfactant production in lung disorders, and for disorders of the urinary bladder epithelium. The compositions of the present invention comprise dehydroepiandrosterone and vitamin A derivatives.

57 Claims, No Drawings

COMBINED DEHYDROEPIANDROSTERONE AND RETINOID THERAPY FOR EPITHELIAL DISORDERS

FIELD OF THE INVENTION

The present invention relates to methods of using dehydroepiandrosterone (DHEA) and its derivatives in combination with vitamin A derivatives (retinoids) for the treatment and prevention of disorders of epithelial tissues.

BACKGROUND OF THE INVENTION

With aging, in both men and women, skin becomes thin, transparent, dry, and prone to uncontrolled growth of its superficial or epidermal layer. Sun exposure produces "photo-aging," which accelerates these changes and stimulates the development of premalignant, raised, roughened areas (actinic keratoses), and malignant tumors (squamous and basal cell carcinomas).

In aging skin, at the microscopic level, the outermost layer of cells, or "stratum corneum," shows diminished cellular cohesion. Furthermore, dry or cornified cells are shed more rapidly than in younger skin. In the deeper granular layer, the predominant cell type, called the keratinocyte, is known to have fewer intracellular keratohyalin granules and fewer subcellular organelles in older skin compared to younger skin.

The lipid-based "epidermal water barrier," which emanates from the granular layer, has been shown to have reduced complex lipid and ceramide content in aged skin when compared to younger skin. The characteristic subcellular organelle of the granular-layer keratinocyte is the "lamellar body" or "keratinosome," which is composed of phospholipids, ceramides, and free sterols. The lamellar bodies migrate to the periphery of the granular cell, where they fuse with the plasma membrane and expel their contents into the intercellular space. Once in the intercellular space, ceramides organize to form the intercellular cement, holding together the more superficial corneal cells and constituting the lipid barrier of the epidermis.

The precursors for biosynthesis of ceramides and specialized lipids include essential fatty acids and the action of subcellular organelles, called "microbodies" or "peroxisomes." Peroxisomes are enzyme-rich and are known to participate in the metabolism of long-chain fatty acids derived from the essential fatty acids.

Both exposure to ultraviolet light and aging are associated with diminished peroxisome numbers and enzymatic activity.

Basal cells form the deepest epidermal cell layer. With aging, they become shorter and less adherent to the dermal layer below. Their nuclei become irregularly shaped, supplying further evidence of disordered growth and differentiation of older, as compared to younger, skin.

Chronic sun exposure predominantly alters the dermis, where elastic fibers thicken, degrade, and degenerate in amorphous masses. Collagen is damaged due to proteolytic enzymes associated with ultraviolet-light-induced inflammation. As a result, wrinkles become deeper and permanent. Also within the dermis, sebaceous glands become larger and contain more cells, even though their production of sebum is less.

Therapy for skin-aging and photo-damage has been advocated to reduce occurrence of epidermal cancer with advancing age. Such therapies have included the use of sun-blocking chemicals, such as para-aminobenzoic acid and methoxy-cinnamate.

Most recently, topical therapy with active vitamin A derivatives, especially retinoic acid, have been shown to specifically stimulate tissue repair in the dermal layer and proliferation and thickening of the more superficial epidermal layers. Retinoid therapy results in a reduction of fine wrinkles, promotion of new collagen synthesis, and reduction of the occurrence of sun-induced basal cell carcinomas. However, retinoic acid therapy often causes dryness, redness, burning, peeling, and increased sun sensitivity of the skin. These side effects make retinoic acid therapy unacceptable to many individuals.

Microscopic study of retinoic acid-treated skin reveals altered keratin protein production, reduced cell-to-cell cohesion, changes in the composition of the lipid barrier of the granular layer, and cellular disorganization of the superficial stratum corneum. An increase in trans-epidermal water loss is seen with retinoid therapy which is similar to that observed in atopic dermatitis and congenital deficiency of the lipid barrier in ichthyosis.

Systemic therapy using oral retinoic acid has proved useful in preventing precancerous changes in the oral mucosa of the elderly ("leukoplakia") and in preventing the development of new tumors in individuals who have had previous squamous cell carcinomas of the head and neck. However, such systemic therapy with retinoids results in similar, or more severe, toxic reactions than those observed with topical use, including pseudo tumor cerebri, hepatitis, dry eye syndrome, hyperostosis, and hyperlipidemia.

A need exists for improved therapy to protect the skin from ultraviolet-light-associated damage and to reduce the toxicity of current retinoid therapy for various epithelial disorders. Preferably, such treatments would stimulate the natural biosynthesis of the epidermal lipid barrier in skin that is lost with aging and diminished as a result of retinoid use.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for the treatment of oxidative epithelial damage, inadequate surfactant production in lung disorders, and disorders of the urinary bladder epithelium. The compositions of the present invention comprise dehydroepiandrosterone (DHEA) and pharmacologically active vitamin A derivatives.

More specifically, the DHEA suitable for use in the present invention is selected from the group consisting of the free alcohol of DHEA, DHEA-S, DHEA derivatives which retain the pharmacologically activity of DHEA, and mixtures thereof, and the vitamin A derivatives are selected from the group consisting of retinoic acid, 13-cis-retinoic acid, N-(4-hydroxyphenyl)retinamide, all-trans-retinoic acid, retinal palmitate, β-carotene, other precursors and derivatives of retinoic acid, vitamin A and vitamin A derivatives, and mixtures thereof.

The DHEA/vitamin A derivative compositions may be formulated for oral, intranasal, topical, intravenous or sublingual administration.

DETAILED DESCRIPTION

The present invention provides a method for combining dehydroepiandrosterone (DHEA) with retinoids to reverse tissue changes due to aging and prevent changes due to ultraviolet light exposure.

During the aging process in most mammals, one of the most dramatic hormonal changes which occurs in both

males and females is the progressive fall in DHEA. DHEA and its sulfated derivative, DHEA-S, comprise the most abundant steroid, released into the circulation by the adrenal cortex. However, starting in the third decade of life, daily secretion of the hormone begins a continuous decline, and by the seventh decade of life, a 90% reduction in DHEA blood levels is commonly observed.

Although the full range of biological activity of DHEA has not been established, it has been shown to play a role in a number of cellular activities in various tissues, such as in sebaceous gland activity in skin; in nervous tissue, as in the treatment of central nervous system degeneration, especially in Alzheimer's Disease; in treatment of diabetes; in treatment of arthritis; in promoting cervical ripening in pregnancy; in anti-carcinogenic activity; in anti-atherosclerotic activity; and in various effects on metabolism in animals, especially a proliferative effect on subcellular peroxisomes.

The composition of the present invention combines the activity of DHEA and retinoids, which combination reduces side effects and toxicity which result from the use of either DHEA or retinoids alone. The side effects of increased sebum production and associated acne, caused by DHEA's stimulation of sebaceous gland tissue, are reduced by the drying effect of retinoids, which reduce the production and release of sebaceous oil. DHEA, likewise, ameliorates the excessive skin dryness, redness, and increased photosensitivity induced by retinoids, by improving the quantity and quality of the ceramide-based lipids produced by the granular keratinocytes. The combinations of DHEA and retinoid may be delivered or administered as a single component, for example, in a single cream, or the combination may be administered separately, for example, a cream with one of the components may be administered, followed by administration of the second component.

The combinations described herein can be used topically or systemically to reduce the changes associated with intrinsic and photo-aging of skin. The combination also has an anti-proliferative, anti-carcinogenic, and differentiation-enhancing effects on other epithelial tissues.

The present invention provides a method for using DHEA and its derivatives and analogues, in combination with retinoids, to restore production of specialized lipid synthesis by keratinocytes in skin and other ectodermally-derived tissue. Specific examples of responsive non-skin keratinocytes include keratinocyte-like cell populations found in the pineal gland and the thymus.

In the present invention, DHEA is used as an agent to stimulate peroxisomes and to prevent or reverse the reduction in peroxisomal activity due to aging-related decreases in DHEA tissue levels. Without being bound by scientific theory, DHEA therapy is thought to stimulate new peroxisome formation to compensate for ultraviolet-light-induced peroxisomal inactivation. An increased peroxisome population provides for increased synthesis of the sphingolipid precursors of ceramides. In addition, stimulated peroxisomal metabolism increases resistance to oxidative stress by increasing the turnover of membrane phospholipids which have been damaged by ultraviolet-light-triggered peroxidation.

DHEA, of the present invention, acts as an agent to stimulate the peroxisomal-based biosynthesis of complex lipids, especially the sphingolipid-derived ceramides. DHEA therapy specifically stimulates keratinocytes and other hormonally-responsive epithelial cells to produce increased amounts of these and related membrane-active, structural lipids. With aging and various abnormal skin conditions, such as atopic dermatitis, psoriasis, and congenital ichthyosis, there is a reduction in epidermal ceramide content associated with scaly and dry skin. The loss of these lipids results in a deficient lipid barrier, permitting increased penetration of environmental agents into the skin and diminished repair of ultraviolet-light-induced damage.

Retinoid therapy results in the proliferative response of dermal and epidermal cell types, which partially reduces photo-aging changes. The toxic effects of retinoids are related to a further reduction in age-related deficiencies in the lipid barrier. Thus, retinoid therapy induces reversal of existing premalignant skin changes but, when combined with ongoing ultraviolet exposure, reduces resistance to oxidative damage, and can serve as a tumor promoter. DHEA counteracts these unwanted actions of retinoids.

The various forms of DHEA useful in the present invention are pharmacologically active DHEA; dehydroepiandrosterone-sulfate (DHEA-S); the free alcohol of DHEA; derivatives of DHEA, such as DHEA 3-acetate (3-hydroxy-5-androsten-17-one-acetate), DHEA-3-glucuronide (3-hydroxy-5-androsten-17-one-3-glucuronide), DHEA-hemisuccinate, DHEA-valerate, DHEA-enanthate, DHEA-fatty acid derivatives, 16-fluorinated, 16-brominated DHEA, and DHEA-salts; and other such DHEA compounds which retain the biological function and activity of DHEA.

Also useful for use in the present invention are metabolic precursors of DHEA, such as pregnenolone, and active metabolites, such as β-etiocholanolone. Other steroid hormones with weak peroxisome-inducing activity but lacking convertability to androgens, such as progesterone, are also useful when avoidance of hair follicle stimulation is necessary to minimize androgenic alopecia.

The various forms of vitamin A for use in the present invention include pharmacologically-active retinal and retinol, but especially retinoic acid (tretinoin). In addition, the highly-active synthetic vitamin A derivatives, such as 13-cis-retinoic acid (iso-tretinoin), all-trans-retinoic acid, N-(4-hydroxyphenyl)retinamide, 4-HPR(fennetinide), and other synthetic vitamin-A derivatives can also be employed, but at lower doses than the naturally-occurring vitamin A derivatives. For cosmetic applications, the less-active, naturally-occurring vitamin A derivatives, such as retinal palmitate and precursor β-carotene, may be used.

Treatments with Retinoid-DHEA Combinations Topical Administration

The retinoid-DHEA combination may, if desired, be administered in appropriate pharmacologically acceptable carriers in such forms as creams, lotions, lipsticks, and dispersible powders.

In such treatments, DHEA and retinoids are solubilized in a suitable carrier, such as cetyl alcohol, glyceryl stearate, polyethylene glycol-100 stearate, sorbitol, water, or combinations thereof. To this basic carrier formulation are added ethylene-diaminetetraacetic acid (EDTA) and vitamin E alcohol as a preservative and an anti-oxidant, respectively, to form a cream base carrier. EDTA is added at a concentration of about 0.05% to about 0.1%. and vitamin E alcohol is added at a concentration of about 0.05% to about 0.1%. The term % as used herein is % weight by volume, unless otherwise stated.

A combined DHEA-retinoid-containing cream (0.75% DHEA, 0.075% retinoid) for topical treatment is prepared by forming an oil-in-water emulsion as follows: The water phase is prepared by mixing 50 mg of EDTA and 5 cc of

propylene glycol in 85 cc of water, and heating to 65° C. for 5 min. The oil phase is then formed by combining 10 cc of cetyl alcohol and 4 cc of 21 stearyl ether, supplied under the name of Brij 721 by Sigma Chemical Co., St. Louis, Mo., Catalog No. P6153, and heating to 60° C. 750 mg of crystalline DHEA alcohol and 7.5 mg of retinoic acid is added to the heated oil phase, with constant mixing, until the DHEA and retinoic acid are dissolved. The water phase is slowly added to the oil phase, with constant mixing. The resulting emulsion is allowed to cool, and 0.1 cc of vitamin E alcohol is added to the cooled mixture. The concentrations can be varied from about 0.001% to about 0.2% for the retinoid, and about 0.05% to about 1.0% for the DHEA (w/v). Preferably, the retinoic-acid concentration is about 1%, and the DHEA concentration is about 0.75%. Application of the mixture is preferably performed at night, prior to sleep, to avoid ultraviolet light when first applied.

The treatment of oxidative epithelial damage of a mammal can be achieved by topical application of DHEA and retinoids in a cream base carrier, as described above. Oxidative epithelial damage is defined, in the present invention, to include the effects of aging on the skin as well as chronic conditions, such as atopic dermatitis, psoriasis, vernal conjunctivitis, congenital ichthyosis, scaly and dry skin, certain dry eye conditions, blepharitis, ultraviolet-induced skin damage, damage caused by chemical peel procedures, superficial burns, chronic papilloma herpes viral infection, and squamous cell carcinoma.

Topical treatment of oxidative epithelial damage can be achieved by using concentrations of DHEA and the retinoid which are at the lower of the range described above and which have been added to a cosmetic base for daytime use. The lower concentrations reduce any irritation to the skin that may result from sun exposure. In such mixtures, the retinoid concentration is about 0.001% to about 0.01%, and the DHEA concentration is about 0.05% to about 0.25%. Preferably, the retinoid concentration is about 0.01%, and the DHEA concentration is preferably about 0.1%. In this embodiment of the present invention, the cream base carrier also comprises ultraviolet-light blocking agents such as zinc oxide, para-aminobenzoic acid, or methyl-cinnamate; vitamin D; and additional lipids such as ceramides. In such mixtures, the ultraviolet-light blocking agents are at a concentration of about 2% to about 7% (octyl methoxy cinnamate) or about 1.5% to about 2.5% (titanium dioxide); the vitamin D is added at a concentration of about 0.001% to about 0.005% (1a-hydroxy vitamin D_3 or $1,25(OH_2)D_3$); and the additional lipids are added at a concentration of about 1% to about 10%. This cosmetic preparation is preferably used by post-menopausal women and is applied to skin prior to sun exposure.

When delivered topically for promotion of the epithelial lipid barrier, DHEA, its related metabolites, and brominated derivatives can all be used in varying concentrations and combinations, either alone or in combination with other steroids such as progesterone and estrogen, which promote ceramide and other specialized lipid biosynthesis. DHEA alcohol, or its metabolite, β-etiocholanolone, is preferably combined with a retinoid, such as tretinoin at a concentration of about 0.1%, and is suitable for use in post-menopausal women. Lower concentrations of DHEA (about 0.1%) are preferably used in combination with lower-concentration retinoids (tretinoin 0.025%), in younger women and in men of all ages, where the natural lipid barrier has been partially maintained and less androgenic activity is desired. The further addition of 5-alpha reductase inhibitors (progesterone, spirono-lactone, and 4-azasteroids [Proscar, Merck]) in concentrations of about 1% to about 5% is preferred to protect against the side effect of androgenic alopecia where such side effects are to be avoided.

When delivered topically to treat intrinsic and photoaging of the skin, the combinations of DHEA and retinoids described herein, may also be use in further combination with other vitamins (E, D, and C); anti-oxidant peptides such as glutathione, ubiquinone, phospholipids (e.g., phosphatidylcholine and phosphatidylserine); and sphingolipids, especially ceramides. Suitable concentrations for use in the present invention are about 0.1% to about 1% of vitamin E; about 0.001% to about 0.005% of vitamin D (1α-hydroxy vitamin D_3 or $1,25(OH_2)D_3$); about 0.5% to about 1% of vitamin C; about 0.01% to about 0.05% anti-oxidant peptides, such as glutathione; about 0.002% to about 0.01% ubiquinone; about 1% to about 10% of phospholipids; and about 1% to about 10% of sphingolipids where such additions may be desirable.

Combinations of DHEA and retinoids can also be used with synergistically-active macrophage stimulators, such as glucans and ace-mananas; or immunomodulators, such as interferons, interferon antagonists, and interleukins; colony-stimulating and growth-stimulating factors, such as transforming growth factor beta (T-GFβ) and epidermal growth factor (EGF); or other hormones such as melatonin. For use in the present invention, glucans are used at a concentration of about 0.01% to about 0.05%; ace-mananas are used at a concentration of about 0.1% to about 0.5%; interferons are used at a concentration of about 0.01% to about 0.2%; interferon antagonists are used at a concentration of about 0.01% to about 0.2%; interleukins are used at a concentration of about 0.01% to about 0.2%; colony-stimulating factors are used at a concentration of about 0.01% to about 0.2%; growth factors (T-GFβ and EGF) are used at a concentration of about 0.01% to about 0.02%; and hormones, such as melatonin, are used at a concentration of about 0.1% to about 0.5%, where such combinations may be desirable.

The above described treatments with DHEA and retinoids alone or in combination with the other described additives will be most appropriate for oxidative damage to epithelial surfaces, which occurs in association with aging-related reductions in circulating DHEA. However, such therapy is also appropriate as a preventative therapy for chronic dermatitis in younger individuals with familial disorders of the epidermal lipid barrier. These disorders include atopic dermatitis, vernal conjunctivitis, psoriasis, and the ichthyoses. Such therapy is additionally appropriate in treating certain acquired disorders of epithelial tissue in all ages, especially chronic viral infection with the Human Papilloma Virus (HPV) and in reducing hypertrophic scar formation following burns, dermabrasion, and chemical peel procedures.

Systemic Administration

The compositions of the present invention may be administered by systemic administration in the form of parenteral, sublingual, transdermal, intranasal, or oral delivery. DHEA and retinoids, as described above, can be incorporated into nanospheres, microspheres, or liposomes for injection, and can be incorporated into cyclodextrins for sublingual and aerosol delivery. All such methods are well known to those skilled in the art.

The retinoid-DHEA combination may, if desired, also be administered in appropriate pharmacologically acceptable carriers in the form of tablets, granules, capsules, syrups and elixirs. The tablets may contain one or more compounds, in

addition to the retinoid-DHEA combination, by admixing with conventional pharmacologically acceptable excipient, such as inert diluents (for example calcium carbonate, sodium carbonate, lactose, sorbitol and talc) granulating and disintegrating agents (such as starch and alginic acid) binding agents (such as sorbitol microcrystalline cellulose, gelatin and acacia) and lubricating agents (such as magnesium stearate, stearic acid and talc). The tablets may be uncoated or coated by known techniques to delay disintegration and adsorption in the gastrointestinal tract or incorporate cyclodextrans to provide a sustained action over a longer period of time.

For oral administration, the DHEA and retinoids are formulated in capsules or in sustained-release tablets. For parenteral administration, the DHEA and retinoid are solubilized in about 5% serum albumin solution with added (1:1 vol:vol) dextran (such as that supplied by Sigma Chemical Co., St Louis Mo., Cat No. D4133). For sublingual administration, the DHEA and retinoids are formulated by solubilizing in a solution of hydroxypropyl-δ-cyclodextrin, then freeze-drying and compressing into tablets. For intranasal administration, the DHEA and retinoids are formulated as for sublingual administration and resuspended in 0.9% saline or a suitable carrier for metered aerosol delivery.

For oral use, the composition of the present invention the dosage rate comprises administering DHEA from about 0.09 g to about 1 g of DHEA per day, with a retinoid of from about 0.025 to 0.2 g of retinoid per day. The treatment is conducted by administering DHEA about 3 times a day and the retinoid, about once a day. The treatment is continued for about four weeks and then adjusted to the minimally-effective dose. In one embodiment of the present invention, the retinoid, for oral use, is isotretinoin (13-cis-retinoic acid).

Treatment of a mammal by systemic administration with the compositions of the present invention is desirable to prevent the recurrence of squamous cell carcinomas in elderly individuals, or, in prematurely-born infants, to stimulate differentiation and increased surfactant production by the alveolar epithelium of the lungs. In this use, the agents specifically stimulate Type-Two alveolar cells, to produce and secrete increased amounts of surfactant. Such treatments supplement the therapy of surfactant-deficiency states, seen in chronic obstructive pulmonary disease in the elderly and in hyaline membrane disease of premature newborns. The compositions of the present invention are also useful for the systemic treatment of proliferative, aging-associated disorders of oral epithelium (leukoplakia) or urinary bladder epithelium (metaplasia), including secondary prevention of transitional cell carcinoma of the bladder.

EXAMPLE 1

Use of DHEA-Retinoid Combinations to Stimulate Differentiation and Increased Ceramide Production in Cultured Human Keratinocytes

Submerged culture of normal human keratinocytes is an established experimental system for the study of the control of differentiation and growth of skin cells. Data on pharmacologic and hormonal control in this system have been found to be directly applicable to cell behavior in intact human squamous epithelium.

The following experiments utilize keratinocyte culture in conjunction with high-performance thin-layer chromatography (HPTLC), to establish the action of DHEA and synergistic combinations of DHEA and retinoids on ceramide production by keratinocytes.

Human epidermal cell cultures (HEK) were established at Clonetics Corporation, San Diego, Calif., according to established procedures. Proliferating cells, from cell line NHEK-346, were subcultured at a density of $2.5\times10^{-3}/cm^2$ in flat culture flasks, in steroid-free, 0.03 mM calcium, keratinocyte growth medium (KGM) supplied by Clonetics Corp. of San Diego. The cells were incubated at 37° C. in an humidified atmosphere of 5% CO_2 in air.

At the start of the experiment, equal aliquots of cells were transferred to eight new flasks containing fresh KGM with a higher calcium concentration (0.15 mM), to permit ongoing differentiation. A test medium was added to each of the flasks. The test media were as follows:

1. KGM only (Control)
2. KGM+0.004% dimethylsulfoxide (DMSO) (diluent control)
3. KGM+0.002% DMSO+0.1 μM DHEA
4. KGM+0.002% DMSO+10 μM DHEA
5. KGM+0.004% DMSO+0.1 μM Retinoic Acid (RA)
6. KGM+0.002% DMSO+10 μM RA
7. KGM+0.004% DMSO+0.1 μM RA+10 μM DHEA
8. KGM+0.004% DMSO+10 μM RA+0.1 μM DHEA

The cells were harvested after an additional 72 hours incubation, counted, and observed microscopically.

Aliquots of equal numbers of viable cells from each of the eight flasks were then separately subjected to lipid extraction as follows: 100 μl of cell suspension was added to 2 ml of ethanol:diethyl ether (3:1 vol:vol), sonicated, and filtered through 0.22-micron hydrophobic microbore filters supplied by Alltex of Nevada City, Calif. The samples were dried under a stream of nitrogen, redissolved in 60 μl chloroform-:methanol (2:1 vol:vol), and frozen under nitrogen at −70° C. until processed further.

Ceramide analysis was performed in a two-step procedure on HPTLC plates (silica 60, 10×20 cm plates supplied by Merck of West Point, Pa. Five μl of the samples were applied onto the plates. The plates were developed to 5.5 cm in solvent system I (methanol:chloroform:water, 20:95:1, by volume) and dried in cool air. The plates were then developed to 9.0 cm in solvent system II (petroleum ether:diethylether:acetic acid, 70:30:10, by volume). Subsequently, they were dried under a stream of warm air.

Lipid spots were visualized by degradative charring. After chromatography, the plates were sprayed with 10% Cu-II-acetate (w/v) in 8% (w/v) o-phosphoric acid and charred for 64 min. at 155° C. in a gaschromatograph oven. Quantification of lipids was by comparison with commercially available lipid standards (supplied by Sigma Chemical Co.) were run on the same plate every time: cholesterol-3-sulfate (Catalog No. C9523), cerebroside type II (Catalog No. C1516), ceramide type III (Catalog No. C2137), ceramide type IV (Catalog No. C2512), cholesterol (Catalog No. C8523), triolein (Catalog No. T4141), cholesteryl oleate (Catalog No. C9253), palmitic acid (P-9767), squalene (S-3626), and n-pentacosan (Catalog No. P7260). Lipid standard mixtures containing 100 ng to 2 μg of each lipid were applied to each plate.

The comparison of the lipids extracted from the treated cells to the lipid standards demonstrated increases in the quantity and variety of ceramide lipids from keratinocytes stimulated by DHEA. Ceramide production was not stimulated by retinoic acid, but the combinations of DHEA and retinoic acid produced a unique pattern of ceramide production, as well as affecting the quantities of other classes of lipids produced.

EXAMPLE 2

Use of the Hairless Mouse to Demonstrate the Topical and Photo-Protective Action of DHEA-Retinoid Combinations

The Skh hairless mouse has been used as a model to study the photo-aging effects of chronic, low-dose ultraviolet (UV) radiation on the skin. There are dramatic changes in the appearance of the skin surface, described as wrinkling and sagging. In addition, tumors develop, consisting of papillomas and carcinomas. Histologically, there are a number of alterations in the epidermis and dermis which are similar to those observed in sun-exposed human skin. For these reasons, the hairless mouse model was used to demonstrate the specific protective effect of DHEA-retinoid combinations in the following protocol.

Female, albino, hairless Skh:HR-1 mice were obtained from Charles Rivers Laboratories. They were 12 weeks old at the start of trials. The mice were housed individually in special radiation cages and had free access to food and water.

Animals were irradiated for 15 min., three times a week for 15 weeks, with a bank of four Westinghouse FS20 sunlamps, placed 45 cm above their backs. Within the UV spectrum, about half the radiation was in the UV-B range (280–320 nm), while the remainder was in the UV-A range (320–400 nm). Less than 1% of the emission was below 280 nm (UV-C range). Flux was measured with an IL 700 A Radiometer (International Light, Newburyport, Mass). The daily doses of UV-B and UV-A were 0.09 J/cm^2 and 0.07 J/cm^2 respectively. Animals were randomly divided into one of five groups of seven mice each and treated with:

1. 100 μl acetone (control)
2. 100 μl acetone+10 μg trans-retinoic acid (RA)
3. 100 μl acetone+10 μg DHEA
4. 100 μl acetone+10 μg DHEA+1 μg RA
5. 100 μl acetone+1 μg DHEA+10 μg RA.

Twenty-four hours prior to each cycle of irradiation, each group received the above treatment, distributed evenly over the skin of the back.

Weekly observations were made on all animals. These observations included uniform scoring as to wrinkle scale for the quantification of tumors and skin appearance. The grading scale is presented in Table I.

TABLE I

Grade	Description of Grading Scales for Visible Skin Changes Description of Skin
	Skin wrinkling
0	Numerous fine striations covering back and sides of body. Fine striations run length of body (head-to-tail direction).
0.5	Loss of fine striations on back along spine. Numerous fine striations along sides run length of body (head-to-tail direction).
1.0	No fine striations on back and along sides. Skin is smooth.
1.5*	No fine striations on back and along sides. Some shallow permanent wrinkles across back (perpendicular to head-to-tail direction).
2.0*	No fine striations on back and along sides. Several shallow to moderately deep permanent wrinkles across back (perpendicular to head-to-tail direction).
2.5*	No fine striations on back and along sides. Numerous moderately deep to deep permanent wrinkles across back (perpendicular to head-to-tail direction).
3.0*	No fine striations on back and along sides. Numerous deep permanent wrinkles across back (perpendicular to head-to-tail direction).
	Skin sagging
0	Numerous fine striations covering back and sides of body. Fine features run length of body (head-to-tail direction). Skin has pale purple-pink coloration.
0.5	Slight reduction in fine striations on back. Numerous fine features along sides run length of body (head-to-tail direction). Slight spotty blanching of skin on back.
1.0	Moderate reduction in fine striations on back and along sides. Slight blanching on skin on entire back.
1.5	Most fine striations gone. Slight nodular wrinkling on back, with no orientation to nodules. Moderate blanching of skin on entire back.
2.0	All fine striations gone. Moderate nodular wrinkling on back with no orientation to nodules. Complete blanching of skin on entire back. Slight loose folds of skin (head-to-tail direction) on sides.
2.5	All fine striations gone. Moderate to severe nodular wrinkling on back, with no orientation to nodules. Complete blanching of skin on entire back. Moderate loose folds of skin (head-to-tail direction) on sides.
3.0	All fine striations gone. Severe nodular wrinkling on back, with no orientation to nodules. Complete blanching of skin on entire back. Large loose folds of skin (head-to-tail direction) on sides.

*The first appearance of tumors is usually at a grade of 1.5. Higher grades are almost always accompanied by tumors.

At 10 and 15 weeks, skin biopsies were performed on representative animals and were subjected to histologic evaluation using Hematoxylin, Eosin, and elastin stain.

Observation of the treated mice demonstrated a photo-protective effect of DHEA-treated mice in whom diminished erythema and diminished tumors were observed. Retinoid-treated mice developed an erythematous dermatitis. DHEA and DHEA-retinoid combination-treated mice developed less dermatitis. The combination DHEA and retinoic acid resulted in mice with the least evidence of photo-aging. The combination of DHEA 10 μg and retinoic acid 1 μg was the most effective in protecting against photo-aging. This combination also resulted in minimal erythema and had the lowest wrinkle index.

EXAMPLE 3

Preparation of a Phospholipid-DHEA-Retinoid Emulsion for Ophthalmic or Dermatologic Use

A DHEA-lipid emulsion was prepared by adding about 500 mg crystalline DHEA and 50 mg crystalline retinoic acid (Sigma, Catalog No. R-2625) to a mixture of about 0.7 ml n-octanoic acid (Sigma, Catalog No. C2875). The mixture was heated to about 55° C. and stirred until the crystals were dissolved, about 10 min.

In a separate container, about 350 mg phosphatidylinositol (Sigma, Catalog No. P5766) and 350 mg phosphatidylglycerol (Sigma, Catalog No. P9524) were added to about 100 ml borate-buffered physiological saline, pH 7.2. Polyquaternium-1 (POLYQUAD, supplied by Alcon Labs., Inc., of Fort Worth, Tex.) was added to a final concentration of about 0.001% to inhibit bacterial growth in the emulsion. The mixture was heated to about 55° C. and stirred to

facilitate the dispersion of the phospholipids, phosphatidylinositol, and phosphatidylglycerol in the saline.

The DHEA-retinoid-octanoic acid mixture, at 55° C., was slowly added to the phospholipid mixture with constant stirring. The mixture was stirred for about 30 min., to form a stable emulsion. The emulsion was then cooled to about 24° C., and about 0.1 ml alphatocopherol acetate (Sigma, Catalog No. T3001) was added with further stirring. The emulsion was transferred to sterile containers prior to use as eyedrops or to application to the skin.

EXAMPLE 4

A glossy, white cream containing DHEA and retinoic acid was prepared as follows (all quantities are wt/wt):

Part	Components	g/100 g
A	cetyl alcohol (CO-1695, Proctor and Gamble Co.),	10.00
	Brij 721	4.00
B	DHEA alcohol,	0.75
	retinoic acid	0.07
C	water (deionized),	79.93
	propylene glycol,	5.00
	EDTA	0.05
D	sodium hydroxide (10% aqueous)	0.10
E	vitamin-E alcohol	0.10

Part A was heated to 60° C., and Part C to 70° C. Part B was added to Part A and mixed until the crystals dissolved. Part C was slowly added to Parts A and B and agitated with an anchor-type stirring blade. Part D was then added, and the mixture was allowed to cool. Water was added to replace that which was lost due to evaporation, and, while the mixture was maintained, the ABCD mixture was cooled to 30° C. Part E was added after the ABCD mixture was cooled to below 30° C. The pH was adjusted to 5.5, if necessary, and the mixture was homogenized. The composition is applied to the skin at night.

EXAMPLE 5

Preparation of a DHEA-Retinoid Cream for Topical Treatment of a Mammal

A cream is prepared by the procedure described in Example 4, with the exception that the concentration of DHEA is reduced to 0.25%, retinoic acid is reduced to 0.01%, and 1.5% titanium dioxide is added to Part C. The resulting cream is applied to the skin during the day.

EXAMPLE 6

Preparation of a DHEA-Retinoid Composition for Intranasal Administration

A solution of hydroxypropyl-δ-cyclodextrin is first prepared by forming a saturated solution of 40 mg of hydroxypropyl-δ-cyclodextrin in 100 cc of water. The solution is decanted and undissolved cyclodextrin discarded. A second solution is made by dissolving 1000 mg of DHEA alcohol and 50 mg of retinoic acid in a minimum sufficient volume of ethanol to dissolve the crystals. The DHEA-retinoic acid, dissolved in ethanol, is added dropwise to the cyclodextrin solution, with constant mixing, at 30° C. Lyophilization, to remove 10% of the volume of the combined solution, is performed to remove most of the ethanol. The resulting DHEA-retinoid-hydroxypropyl-δ-cyclodextrin solution is administered nasally or orally by aerosolization.

EXAMPLE 7

Preparation of a DHEA Tablets for Sublingual and Oral Use

A solution of DHEA dissolved in gamma-cyclodextrin is prepared by the method described in Example 6. The sample is lyophilized to dryness, resulting in a white crystalline powder of the mixture of DHEA, retinoid, and cyclodextrin. The mixture is compressed into tablets for sublingual use. The tablets are dissolved under the tongue 3 times daily, to deliver up to 300 mg of DHEA and 15 mg of retinoic acid per day.

EXAMPLE 8

Preparation of a DHEA-Retinoid Combination for Oral Administration to a Mammal

Tablets are prepared by the procedure described in Example 7. The equivalent of 100 mg of DHEA and 5 mg of retinoic acid is administered orally 3 times a day.

EXAMPLE 9

Preparation of a DHEA-Retinoid Combination for Intravenous Administration to a Mammal

A solution of DHEA, retinoic acid, and hydroxypropyl-δ-cyclodextrin is prepared by the procedure described in Example 6. The solution is filtered through a 0.2-micron filter, at 30° C. The resulting sterile filtrate is administered intravenously, using standard techniques, to deliver 50 mg of DHEA and 2.5 mg retinoic acid, 3 times a day, to an adult, and 2 mg/kg DHEA and 0.01 mg/kg retinoic acid to infants and children.

EXAMPLE 10

Additional DHEA-Retinoid Preparation for Intravenous Administration to a Mammal

Twenty mg of retinoic acid and 1000 mg of DHEA are dissolved in 1 cc of ethanol. This solution is added dropwise to a solution of 500 cc of 5% albumin and 500 cc of dextran, with constant mixing. 100% nitrogen is bubbled for 6 hrs. through the resulting suspension, with constant mixing. The suspension is aerated for 6 hrs. with 100% nitrogen, to drive off residual ethyl alcohol. The suspension is then filtered through a 0.2-micron filter using sterile techniques. Ten cc of this solution is administered intravenously 3 times daily to an infant, or, for an adult, 100 cc is administered intravenously 3 times daily.

The above description of exemplary embodiments of methods for treating epithelial disorders using combinations of DHEA and retinoids are for illustrative purposes. Variations will be apparent to those skilled in the art; therefore, the present invention is not intended to be limited to the particular embodiments described above. Also, the invention disclosed may be practiced in the absence of any element which is not specifically disclosed in the specification. The scope of the invention is defined in the following claims.

What is claimed is:

1. A composition for the treatment of epithelial damage in a mammal comprising a pharmacologically active retinoid and pharmacologically active DHEA wherein the combination is effective in preventing or reversing damage to the epithelium due to environmental oxidative agents and wherein the proportion of retinoid and DHEA is sufficient to overcome the undesirable side effects of each composition when used alone.

2. A composition as recited in claim 1 wherein the retinoid is selected from the group consisting of retinoic acid, 13-cis-retinoic acid, N-(4-hydroxyphenyl)retinamide, all-trans-retinoic acid, retinal palmitate, β-carotene, other precursors and derivatives of retinoic acid, vitamin A and vitamin A derivatives, and mixtures thereof.

3. A composition as recited in claim 1 wherein the DHEA is selected from the group consisting of the free alcohol of DHEA, DHEA-S, DHEA derivatives which retain the pharmacological activity of DHEA, and mixtures thereof.

4. A composition as recited in claim 1 wherein the retinoid is present at a concentration of about 0.01% to about 0.5%.

5. A composition as recited in claim 1 wherein the DHEA is present at a concentration of about 0.1% to about 1%.

6. A composition as recited in claim 1 wherein the composition is suitable for oral administration.

7. A composition as recited in claim 6 wherein the composition further comprises hydroxypropyl-δ-cyclodextrins.

8. A composition as recited in claim 1 wherein composition is suitable for intranasal administration.

9. A composition as recited in claim 8 wherein the composition further comprises cyclodextrins.

10. A composition as recited in claim 8 wherein the composition comprises about 1% of DHEA, about 0.05% of retinoic acid, and about 0.4% of hydroxypropyl-δ-cyclodextrin.

11. A composition as recited in claim 1 wherein the composition is suitable for topical administration.

12. A composition as recited in claim 11 wherein the composition further comprises additives selected from the group consisting of vitamin A, vitamin D, vitamin C., antioxidant peptides, ubiquinone, nucleotides, phospholipids, sphingolipids, macrophage stimulators, growth factors, immunomodulators, hormones, ultraviolet-light blockers, carriers, and mixtures thereof.

13. A composition as recited in claim 12 wherein the composition comprises about 0.75% DHEA, about 0.1% retinoic acid, and about 2% titanium dioxide in a cream base.

14. A composition as recited in claim 1 wherein the composition is suitable for intravenous administration.

15. A composition as claimed in claim 14 wherein the composition further comprises serum albumin and dextran.

16. A composition as recited in claim 14 wherein the composition comprises about 0.1% DHEA, about 0.05% retinoic acid, and about 5% serum albumin.

17. A composition as recited in claim 1 wherein the composition is suitable for sublingual administration.

18. A composition as recited in claim 17 wherein the composition further comprises a carrier such as hydroxypropyl cyclodextrin derivatives.

19. A composition as recited in claim 17 wherein the composition comprises about 1% DHEA, about 0.05% retinoic acid, and about 0.04% hydroxypropyl-δ-cyclodextrins.

20. A composition as recited in claim 1 wherein the composition is suitable for administration in the form of eyedrops.

21. A composition as recited in claim 20 wherein the composition further comprises phospholipids.

22. A composition as recited in claim 1 wherein the epithelial damage is selected from the group consisting of disorders of the skin, oral epithelium and disorders of the bladder epithelium.

23. A method for treating epithelial damage in a mammal comprising administering a pharmacologically active retinoid in an amount sufficient to counteract undesirable side effects of administered DHEA and a pharmacologically active DHEA in an amount sufficient to counteract undesirable side effects of administered retinoid to the mammal wherein the combination is effective in preventing or reversing damage to the epithelium due to environmental oxidative agents.

24. A method as recited in claim 23 wherein the treatment is administered topically.

25. A method as recited in claim 24 wherein the retinoid is administered in a dose of about 0.1%, three times daily, and the DHEA is administered in a dose of about 1%, three times daily.

26. A method as recited in claim 23 wherein the treatment is administered intravenously.

27. A method as recited in claim 26 wherein the retinoid is administered in a dose of about 1 mg, three times daily, and the DHEA is administered in a dose of about 30 mg, three times daily.

28. A method as recited in claim 23 wherein the treatment is administered orally.

29. A method as recited in claim 28 wherein the retinoid is administered in a dose of about 20 mg, three times daily, and the DHEA is administered in a dose of about 150 mg, three times daily.

30. A method as recited in claim 23 wherein the treatment is administered sublingually.

31. A method as recited in claim 30 wherein the retinoid is administered in a dose of about 20 mg, three times daily, and the DHEA is administered in a dose of about 150 mg, three times daily.

32. A method as recited in claim 23 wherein the treatment is administered intranasally.

33. A method as recited in claim 32 wherein the retinoid is administered in a dose of about 10 mg, three times daily, and the DHEA is administered in a dose of about 100 mg, three times daily.

34. A method as recited in claim 23 wherein the treatment is administered in the form of eye drops.

35. A method as recited in claim 23 wherein the epithelial damage is selected from the group consisting of disorders of the skin, oral epithelium and disorders of the bladder epithelium.

36. A method as recited in claim 23 wherein the retinoid is selected from the group consisting of retinoic acid, 13-cis-retinoic acid, N-(4-hydroxy-phenyl)retinamide, all-trans-retinoic acid, retinal palmitate, β-carotene, other precursors and derivatives of retinoic acid, vitamin A and vitamin A derivatives, and mixtures thereof.

37. A composition for the treatment of inadequate surfactant production in the lungs of a mammal comprising a pharmacologically active retinoid and a pharmacologically active DHEA.

38. A composition as recited in claim 37 wherein the retinoid is selected from the group consisting of retinoic acid, 13-cis-retinoic acid, all-trans-retinoic acid, retinal palmitate, β-carotene and other precursors of retinoic acid, vitamin A and vitamin A derivatives, and mixtures thereof.

39. A composition as recited in claim 37 wherein the DHEA is selected from the group consisting of the free alcohol of DHEA, DHEA-S, DHEA derivatives which retain the pharmacological activity of DHEA, and mixtures thereof.

40. A composition as recited in claim 37 wherein the retinoid is present at a concentration of about 0.01% to about 0.5%.

41. A composition as recited in claim 37 wherein the DHEA is present at a concentration of about 0.1% to about 1%.

42. A composition as recited in claim 37 wherein the composition is suitable for oral administration.

43. A composition as recited in claim 42 wherein the composition further comprises cyclodextrin derivatives.

44. A composition as recited in claim 42 wherein the composition is suitable for intravenous administration.

45. A composition as recited in claim 44 wherein the composition further comprises about 5% serum albumin.

46. A composition as recited in claim 45 wherein the composition further comprises liposomes.

47. A composition as recited in claim 45 wherein the composition further comprises cyclodextrin derivatives.

48. A method for treating inadequate surfactant production in the lungs of a mammal comprising administering a pharmacologically active retinoid and pharmacologically active DHEA.

49. A method as recited in claim 48 wherein the treatment is administered intravenously.

50. A method as recited in claim 49 wherein the retinoid is administered in a dose of about 0.01 mg/kg, three times daily, and the DHEA is administered in a dose of about 2 mg/kg, three times daily.

51. A method as recited in claim 48 wherein the treatment is administered orally.

52. A method as recited in claim 51 wherein the retinoid is administered in a dose of about 1 mg, three times daily, and the DHEA is administered in a dose of about 150 mg, three times daily.

53. A method for counteracting the toxicity of retinoid administration comprising administering a pharmacologically active DHEA to a patient to whom a retinoid is administered as a mode of treatment, wherein the DHEA is administered in an amount sufficient to counteract the toxic effects of the administered retinoid.

54. A method as recited in claim 53 wherein the DHEA is selected from the group consisting of the free alcohol of DHEA, DHEA-S, DHEA derivatives which retain the pharmacological activity of DHEA, and mixtures thereof.

55. A method as recited in claim 53 wherein the retinoid is selected from the group consisting of retinoic acid, 13-cis-retinoic acid, N-(4-hydroxy-phenyl) retinamide, all-trans-retinoic acid, retinal palmitate, β-carotene, other precursors and derivatives of retinoic acid, vitamin A and vitamin A derivatives, and mixtures thereof.

56. A method as recited in claim 53 wherein the mode of administering the pharmacologically active DHEA is selected from the group consisting of topical, intravenous, oral, sublingual, intranasal and by eye drops.

57. A method as recited in claim 53 wherein the DHEA is administered in a dose of about 30 mg to about 500 mg per day.

* * * * *

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1
PREPARATION OF LIPOSOMAL 4-HPR

To demonstrate the simplified production of a 4-HPR-liposome composition, 4-HPR was mixed with lipid mixture composed of various ratios of dimyristoyl-phosphatidylcholine (DMPC) and soybean oil in tertiarybutyl alcohol-water mixture (Table 2).

Table 2
Incorporation efficiency of 4-HPR in liposomes

Composition of liposome	**4-HPR: Lipid (w/w)**	1 : 17	1 :10				1 : 5	1 : 15
	DMPC: soybean oil in lipid mixture	1 : 0	1 : 0	9 : 1		8 : 2	9 : 1	9 : 1
	Water in tertiary butyl alcohol	0	1	1	10	1	10	10
Incorporation efficiency (%)		60.0	81.5	87.5	92.8	77.5	88.3	96.4

The mixture was frozen in acetone-dry ice bath, and then dried by lyophilzer. It was stored as a powder and resuspended with saline before use.

To determine the incorporation efficiency of 4-HPR into liposomes, liposomal 4-HPR powder was resuspended in saline and any free, unincorporated free 4-HPR was separated by centrifugation at 30,000 xg for 1 hour. Liposomal 4-HPR was collected as a

pellet and washed three times with saline. 4-HPR concentration was determined by absorbance at 345 nm. To remove the interference by liposomal turbidity, absorbance was measured after liposomal 4-HPR before and after separation was diluted with 1:1 mixture of dimethylsulfoxide and water. The incorporation efficiency was calculated by the following equation:

Incorporation efficiency = OD345 (after separation)/OD345 (before separation) X 100%

EXAMPLE 2
ANTI-TUMOR ACTIVITY OF LIPOSOMAL 4-HPR

Anti-tumor activity of liposomal 4-HPR was demonstrated in breast cancer cell lines in terms of growth inhibition (Table 3A and 3B). Breast cancer cell lines were plated in 96 well plates in DMEM/F12 medium supplemented with 5% fetal calf serum and incubated with different concentrations of liposomal 4-HPR and free 4-HPR, respectively. At the end of incubation, the growth of cells was determined by the Celltiter 96 Aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's protocol. Growth and viability of cells were determined by MTS assay after treatment with 4-HPR for 3 days (BT-474) and 4 days (MCF7). Growth of treated cells was compared with that of untreated cells cultured under the same conditions, and was expressed as % of untreated cells. Tables 3A and 3B shows the resulting data, which represents at least 3 experiments for each number.

Table 3A
Comparison of the inhibitory effect of free- and liposomal 4-HPR on the growth and viability of MCF-7 cells

Concentration of 4-HPR (μM)	Growth and viability (%) after treatment with	
	Free 4-HPR	Liposomal 4-HPR
0.1	106.5±7.8	112.7±2.4
0.9	77.5±5.8	72.9±2.9
1.5	43.8±5.7	49.2±3.6
1.8	33.6±2.	35.0±2.6